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p21 regulates common activation pathways in macrophages and T cells and acts as a suppressor of inflammatory and autoimmune syndromes

TESIS DOCTORAL

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ABBREVIATIONS

AICD	activation-induced cell death
ANOVA	analysis of variance
AP-1	activator protein 1
APC	antigen-presenting cell
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BMDM	bone marrow-derived macrophages
CBP	CREB-binding protein
CCL	CC chemokine ligand
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CM-H_2DCFDA	chloromethyl-2',7'-dichlorodihidrofluorescein diacetate acetyl ester
ConA	concavalin A
CoQ	coenzyme Q (ubiquinone)
CRAC	calcium release-activated channel
CREB	cAMP response element-binding protein
Ctrl	control
CXCL	CXC chemokine ligand
Cyt c	cytochrome c
DC	dendritic cell
DNA	deoxyribonucleic acid
DPI	diphenyleneiodonium
e^-	electron
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
Egr2	early growth response 2
EMSA	electromobility shift assay
ERK	extracellular signal-regulated kinase
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FAD	flavin adenine dinucleotide

FADD	Fas-associated death domain
$FADH_2$	flavin adenine dinucleotide (hydroquinone form)
FasL	Fas ligand
FBS	fetal bovine serum
GADD	growth arrest and DNA damage
GM-CSF	granulocyte-macrophage colony-stimulating factor
H^+	hydrogen ion
H_2O	water
H_2O_2	hydrogen peroxide
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
IFN	interferon
IFNAR	IFN-α/β receptor
Ig	immunoglobulin
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRAK	IL-1 receptor-associated kinase
IRF3	IFN-regulatory factor 3
IS	immunological synapse
Jak2	Janus kinase 2
JNK	c-Jun N-terminal kinase
Lck	lymphocyte-specific protein tyrosine kinase
lpr	lymphoproliferation
LPS	lipopolysaccharide
M1	classically activated macrophage
M2	alternatively activated macrophage
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger RNA

mROS	mitochondrial ROS
MyD88	myeloid differentiation primary response protein 88
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
Nrf2	nuclear factor erythroid 2-related factor 2
O_2	molecular oxygen
O_2^-	superoxide
OH^-	hydroxyl radical
OVA	ovalbumin
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
P_i	inorganic phosphate
PI	propidium iodide
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel elecrtophoresis
SEM	standard error of the mean
SHIP	src homology 2-containing inositol-5'-phosphatase
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SOCS1	suppressor of cytokine signaling 1
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
TAK1	TGF-β-activated kinase 1
TBE	tris borate EDTA
TCA	tricarboxylic acid
TCR	T cell receptor

tg	transgenic
TGF	transforming growth factor
Th	T helper
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tol	tolerized
TRAF6	TNF receptor-associated factor 6
TRAPS	tumor necrosis factor receptor-associated periodic syndrome
TRIF	TIR-domain-containing adaptor inducing IFN-β
Treg	regulatory T cell
Tyk2	Tyrosine kinase 2
WT	wild type

ABSTRACT

Proinflammatory and anti-inflammatory immune reactions must be regulated precisely to maintain balance in the immune system. Excessive activation can result in hypersensitivity diseases such as autoimmunity or septic shock, leading to multiple organ dysfunction syndrome and death. Excessive anti-inflammatory activity is also harmful, as it can lock monocytes and macrophages into an unresponsive state. Here we identify a role for p21 as a negative regulator of activated T cells and macrophages, which is independent of the previously known p21 function as a cell cycle inhibitor. Our data show that p21 controls T cell activation and IFN-γ production through negative regulation of mitochondrial reactive oxygen species (mROS) production. When we overexpressed p21 in T cells of *lpr* mice, we were able to reduce T cell production of IFN-γ, which led to amelioration of lupus-like disease in these mice. Further, we show that p21 negatively regulates LPS (lipopolysaccharide)-induced mROS production in macrophages. In the absence of p21, LPS-activated macrophages showed strong NFκB activation and increased IFN-β secretion. As a consequence, p21-deficient macrophages showed impaired ability to reprogram their proinflammatory status to immunosuppression, known as LPS tolerance. Our findings point to a role for p21 in regulating the balance between active p65/p50 and inhibitory p50/p50 NF-κB complexes during LPS tolerance in macrophages. Finally, we show that p21 is an important component of macrophage reprogramming in human monocytes, with implications in sepsis, and thus demonstrate the biological significance of our data.

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DEDICATION

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INTRODUCTION

Immune response: balancing activation and deactivation

To establish an infection, a pathogen must first overcome epithelial barriers, such as the skin and mucus-lined body cavities. Any organism that breaks through this first barrier encounters the next two levels of defense: the innate and adaptive immune systems. Infection by a pathogen triggers an acute inflammatory response in which cells and molecules of the immune system move into the affected site. Substances released by the pathogen and from damaged tissue increase local vascular permeability and upregulate the expression of adhesion molecules on the vascular endothelium, attracting circulating cells to the infection site.

Neutrophils are the first cells that accumulate at the infection site, where they phagocytose the microbe coated with proteins of the complement system. Monocytes and macrophages are recruited to the site shortly after neutrophils, and persist for long periods at sites of chronic inflammation and infection. They produce nitric oxide as a major mechanism for killing bacteria, as well as large amounts of cytokines such as IL12 and IL-18, giving them a regulatory role in adaptive immune responses. By taking up microbial antigens and presenting them in a complex with MHC, macrophages and dendritic cells also have key roles in adaptive immunity, serving as antigen-presenting cells.

T lymphocytes can be divided into different subpopulations with distinct functions. $CD4^+$ T helper cells (Th) support the functions of other immune cells, such as B cells (which produce antibodies), macrophages, and T cells. $CD8^+$ cytotoxic T cells kill cells that express foreign antigens, such as virus-infected cells or tumor cells. Naïve $CD4^+$ T cells can differentiate into a variety of subsets, including Th1, Th2, Th17 and regulatory T cells (Treg). These subsets differ in the cytokines they produce and in their functions in host defense. Th1 cells secrete high levels of IFN-γ and mediate defense against intracellular microbes. Th2 cells secrete high IL-4, IL-5 and IL-13 levels, and mediate defense against helminths. Th17 cells produce IL-17 and IL-22 and defend against extracellular bacteria and fungi. Treg cells suppress immune responses and have an important function in preventing immune reactions against self-antigens.

After the pathogen is eliminated, the majority of effector lymphocytes die by apoptosis, thus returning the immune system to homeostasis. Imbalance between the effector mechanisms of immune responses and the control mechanisms that serve to limit such responses, lead to development of hypersensitivity disease such as allergic and autoimmune disorders. For example, after repeated exposure to an antigen, Th1 cells secrete IFN-γ, which is responsible for many of the manifestations of autoimmune disease. IFN-γ-activated macrophages secrete more cytokines, which promote inflammation, and produce more IL-12, thereby amplifying Th1 responses. Sustained activation thus results in continued inflammation and tissue injury. Furthermore, excessive activation can result in septic shock, multiple organ dysfunction syndrome, and death. In contrast, excess anti-inflammatory activity is also harmful and could lock monocytes and macrophages in an unresponsive state. Proinflammatory and anti-inflammatory immune reactions must therefore

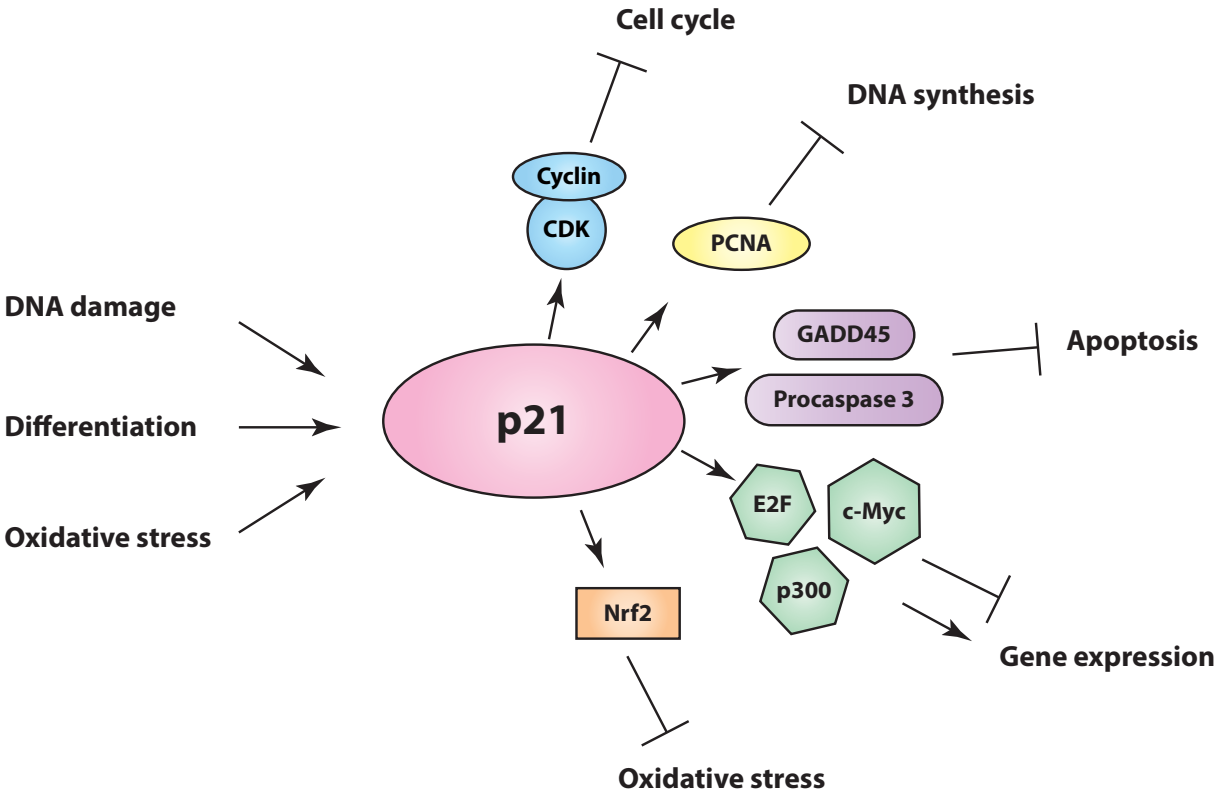
be precisely regulated to maintain immune system balance.

p21$^{(WAF1/Cip1)}$: far beyond cell cycle regulation

p21 (also known as p21$^{WAF1/Cip1}$), a small 165-amino-acid protein, was the first cyclin-dependent kinase (CDK) inhibitor to be identified (Harper et al., 1993). p21 was first described as a mediator of p53-induced G1 arrest in response to DNA damage (Deng et al., 1995; Brugarolas et al., 1995; el-Deiry et al., 1993), as it halts DNA replication until errors are fixed. Today, we know that p21 is involved in a far more complex network of interactions, thus affecting a plethora of biological functions and mechanisms in addition to cell cycle control (Scheme 1).

Non-overlapping structural domains of p21 are responsible for interacting with two targets essential for cell cycle progression. Through its N-terminal domain, p21 interacts specifically with the cyclin-CDK complex, thus preventing phosphorylation of the retinoblastoma protein and the consequent expression of E2F-dependent genes, necessary for cell cycle progression (Vidal and Koff, 2000). p21 can also inhibit DNA replication directly, through its interaction with PCNA (proliferating cell nuclear antigen) via its C-terminal domain (Li et al., 1994; Chen et al., 1995). p21 participates in a number of other protein-protein interactions, which opens the possibility of yet-undiscovered functions of this molecule.

p21 associates with E2F and thus directly regulates gene expression independently of cyclin/CDK (Delavaine and La Thangue, 1999). Through its C-terminal domain, p21 can also bind to c-Myc and suppress c-Myc-dependent



SCHEME 1 | **p21 modulates multiple biological functions.** The ability of p21 to engage in complex network of protein-protein interactions provides a likely basis for novel biological functions yet to be discovered.

gene expression (Kitaura et al., 2000). Besides these transcription factors, p21 can interact with transcription coactivators such as CBP or p300, and enhance their function (Perkins et al., 1997; Snowden et al., 2000). GADD45, another molecule involved in growth arrest and DNA stability, also associates directly with p21 (Smith et al., 1994; Kearsey et al., 1995). Since both GADD45 and p21 can associate with mitogen-activated protein kinase (MAPK)-related proteins, it was proposed that p21 and GADD45 cooperate in control of apoptosis rather than growth arrest (Shim et al., 1996; Takekawa and Saito, 1998). In support of its role in inhibiting apoptosis, p21 can associate with procaspase 3 and block its cleavage and activation, which protects from Fas-induced apoptosis (Suzuki et al., 1998). Paradoxically, some studies indicate that p21 can also promote apoptosis (Hingorani et al., 2000; Fotedar et al., 1999). These contrasting views might be explained by considering that the p21 effect on apoptosis could be dependent on cell type and post-translational modulations such as phosphorylation, which affects p21 cytoplasmic localization (Child and Mann, 2006), or ubiquitylation, which affects p21 stability. Growth-inhibitory and pro-apoptotic p21 functions might thus be associated with its nuclear localization, whereas p21 anti-apoptotic activities might be linked to its cytoplasmic accumulation (Coqueret, 2003).

Besides cell cycle control and apoptosis, p21 has a role in differentiation. In epithelial cells, p21 is downmodulated along the differentiation pathway; conversely, p21 overexpression in these cells inhibits differentiation, suggesting that p21 must be inactivated for the late stages of differentiation to occur (Gartel et al., 1996; Pontén et al., 1995). The role of p21 in differentiation is not linked to its effects on the cell cycle (Di Cunto et al., 1998), and it can be negative or positive depending on cell type and differentiation stage (Casini and Pelicci, 1999). Recent evidence suggests that p21 is important for restricting stem cell self-renewal in keratinocyte and hematopoietic systems, and for promoting their irreversible commitment to differentiation (Cheng et al., 2000; Topley et al., 1999). Absence of p21 therefore leads to increased stem cell potential of keratinocytes, resulting in enhanced susceptibility to carcinogenesis (Topley et al., 1999). In hematopoietic system, however, p21 has a dual role: on the one hand, it inhibits stem cell proliferation, and on the other, it stimulates proliferation of progenitor cells (Cheng et al., 2000).

p21 came into the spotlight as a tumor suppressor after its initial discovery as a potential mediator of p53 tumor suppressor activity (el-Deiry et al., 1993). Although many human cancers correlate with p21 downregulation, p21 loss-of-function mutations are extremely rare, which suggests that p21 itself is not a classical tumor suppressor (Shiohara et al., 1994; McKenzie et al., 1997; Abbas and Dutta, 2009). Additional *in vivo* evidence indicating that loss of p21 is not sufficient to promote malignancy came from a study in $p21^{-/-}$ mice, which showed that these mice do not develop spontaneous tumors early in life, as described for mice deficient in other tumor suppressor genes (Martín-Caballero et al., 2001).

Finally, several studies proposed a role for p21 in regulating oxidative stress (Fan et al., 1997; Esposito et al., 1998). In the lung, hyperoxia induces p21 expression and protects mice from oxidative stress (O'Reilly et al., 2001). p21 was shown to mediate anti-oxidant response through direct interaction with the transcription factor Nrf2 (Chen et al., 2009). Based on this study, p21-mediated induction of Nrf2 signaling was proposed to be a first line of defense used to reduce ROS in low-stress conditions, whereas high oxidative stress would lead to p21-mediated

cell cycle arrest or apoptosis (Villeneuve et al., 2009). In addition, $p21^{-/-}$ mice fed with a high fat diet showed severe atherosclerosis due to increased NADPH oxidase activity, arguing for the protective role of p21 against oxidative stress (Khanna, 2009).

p21 as a regulator of innate and adaptive immune responses

Rather than developing tumors, $p21^{-/-}$ mice unexpectedly show manifestations of autoimmune disease (Balomenos et al., 2000). p21 deficiency combined with mild autoreactive genetic backgrounds such as 129/Sv × C57BL/6 or Gadd45a-deficient mice show severe lupus-like autoimmunity, including splenomegaly and lymphadenopathy, which leads to death (Balomenos et al., 2000; Salvador et al., 2002). In mice on the autoimmunity-resistant C57BL/6 (B6) background, p21 deficiency results in autoantibody production and mild, non-lethal glomerulonephritis, suggesting that full-blown lupus development requires p21 deletion as well as elements from the 129/Sv × C57BL/6 background (Arias et al., 2007). The role of p21 in suppressing autoimmunity was reinforced by study of Egr-2-deficient mice, which show p21 downregulation in T cells and develop autoimmunity (Zhu et al., 2008). Several p21 alleles are associated with the development of systemic lupus erythematosus in humans (Kong et al., 2007). A peptidyl mimic of p21 inhibits progression of lupus-like syndrome in NZB × NZW mice (Goulvestre et al., 2005), suggesting that p21 can serve as a target molecule for improvement of therapeutic strategies for autoimmune disease. Indeed, we found that p21 overexpression in T cells reduces lupus-like disease in *lpr* mice (Daszkiewicz et al., 2015).

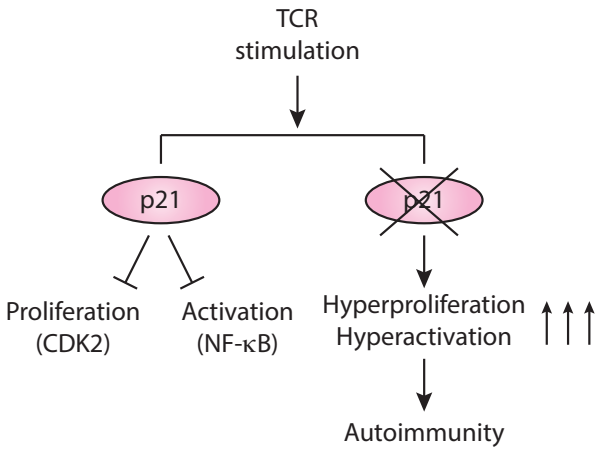
In addition to autoimmunity, $p21^{-/-}$ mice show enhanced proinflammatory cytokine production and septic shock-induced death when challenged with bacterial LPS (Trakala et al., 2009; Scatizzi et al., 2009; Mavers et al., 2012). These reports, including the data from our laboratory, demonstrate a critical role for p21 in regulation of gene expression in macrophages, through negative regulation of NF-κB activation (Trakala et al., 2009). Due to uncontrolled macrophage activation, $p21^{-/-}$ mice also show enhanced development of experimental inflammatory arthritis, which can be suppressed by administration of a p21 mimetic peptide (Mavers et al., 2012). p21 thus negatively regulates the macrophage inflammatory response, and might be of use for controlling sepsis in humans.

Loss of tolerance and (de)regulation of T cell activation, proliferation and apoptosis

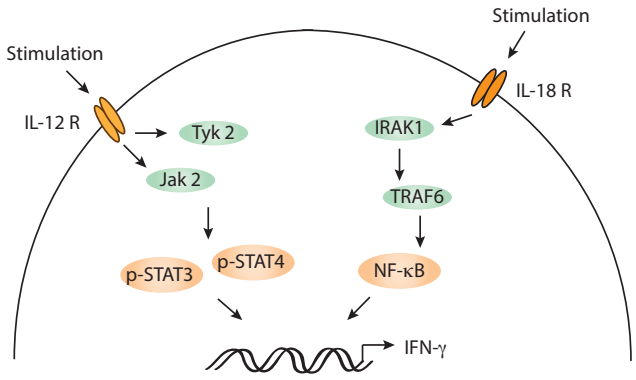
During primary immune responses, mature T cells, characterized by a naïve phenotype (low CD44 and high CD62L expression) recognize a specific antigen and differentiate into highly activated effector cells, which proliferate massively to establish a rapid immune response (Sprent and Surh, 2011). After resolution of the primary response, the majority of effector cells undergo apoptosis, leaving a small population of memory cells (characterized by high CD44 expression) that revert to a resting state (McKinstry et al., 2010). The precise regulation of total cell number, the proportion of naïve and memory T cells, and the extent of memory T cell expansion —known as T cell homeostasis— basically relies on the balance between proliferation and apoptosis (Freitas and Rocha, 2000). Deregulation of any of these steps (activation, proliferation or apoptosis) leads to loss of self-tolerance and autoimmunity.

One way to control autoreactive T cells is activation-induced cell death (AICD). Repetitive TCR engagement with self-antigens triggers AICD through a mechanism that involves Fas/Fas ligand (FasL) interaction and subsequent recruitment of the Fas-associated death domain (FADD) adaptor protein, which activates caspase-8 and triggers the apoptotic cascade (Walker and Abbas, 2002). MRL/*lpr* and *gld* mice, which bear mutations in Fas and FasL respectively, show a lupus-like lymphoproliferative syndrome (Watanabe-Fukunaga et al., 1992; Sobel et al., 1993). Although the apoptotic defect is easy to demonstrate in Fas-deficient T cells activated *in vitro*, whether this defect is responsible for the loss of self-tolerance and development of autoimmunity remains a mystery. The first evidence that defective apoptosis alone cannot account for T cell accumulation came from studies with mice that overexpress caspase-8 inhibitor in T cells (Smith et al., 1996). T cells from these mice showed defective apoptosis *in vitro*, but the mice did not develop lymphadenopathy or autoimmune disease. This suggested that Fas might have another role in T cell homeostasis other than apoptosis regulation. An additional property of Fas emerged from studies showing that T cell subsets from *lpr* mice exhibit a hyperactivation state and hyperproliferate (Balomenos et al., 1997; Fortner and Budd, 2005; Gupta et al., 2008). Autoimmune lymphoproliferative syndrome patients, who bear a mutation in the Fas-encoding gene, similarly show T cell hyperproliferation and lymphadenopathy (Le Deist et al., 1996). Another property of *lpr* T cells is high production of IFN-γ, a Th1 cytokine that modulates anti-DNA antibody production and disease development in several lupus models (Altman et al., 1981; Theofilopoulos et al., 2001). It is thus tempting to speculate that non-apoptotic Fas functions control *lpr* T cell proliferation and activation (Alderson et al., 1993; Kennedy et al., 1999; Bosque et al., 2008).

T cell activation and proliferation are linked, and there are many examples that clearly associate T cell hyperactivation and hyperproliferation with loss of self-tolerance and autoimmunity (Jury et al., 2010; Okazaki and Honjo, 2006; Sharpe et al., 2007). Data from our laboratory provided evidence that T cell homeostasis, tolerance and lupus-like disease are very dependent on cell cycle regulation of effector/memory T cells (Scheme 2). Studies of $p21^{-/-}$ mice suggested that p21 controls the proliferation of effector/memory but not naïve T cells (Balomenos et al., 2000; Arias et al., 2007). Moreover, absence of p21 does not affect Fas/FasL-mediated apoptosis (Arias et al., 2007). T cells nonetheless require p21 to control their expansion, revealing a homeostatic mechanism that controls memory T cell expansion through p21. This role for p21 is in accordance with the fact that hyperproliferating T cells from lupus patients express markedly reduced p21 levels (Tang et al., 2009). A recent study showed that T cell-specific p21 overexpression



SCHEME 2 | p21 suppresses autoimmunity by regulating T cell homeostasis. Studies from our group identify p21 as a negative regulator of effector/memory T cell proliferation. However, independently of its cell-cycle inhibitor function, p21 regulates T cells activation, most likely through NF-κB (unpublished observations).



SCHEME 3 | Synergy between IL-12 and IL-18 for IFN-γ promoter activation. IL-12 receptor stimulation induces phosphorylation of Jak2 and Tyk2, which results in STAT4 activation and its translocation to the nucleus. IL-8 receptor signals through IRAK1 and TRAF6 and results in activation of NF-κB.

reduces lupus-like disease in *lpr* mice by limiting effector/memory T cell activation and proliferation, without affecting the apoptosis defect in these mice (Daszkiewicz et al., 2015). This suggested that p21 has another function in regulating T cell activation, independent of its cell cycle inhibitor role (Scheme 2).

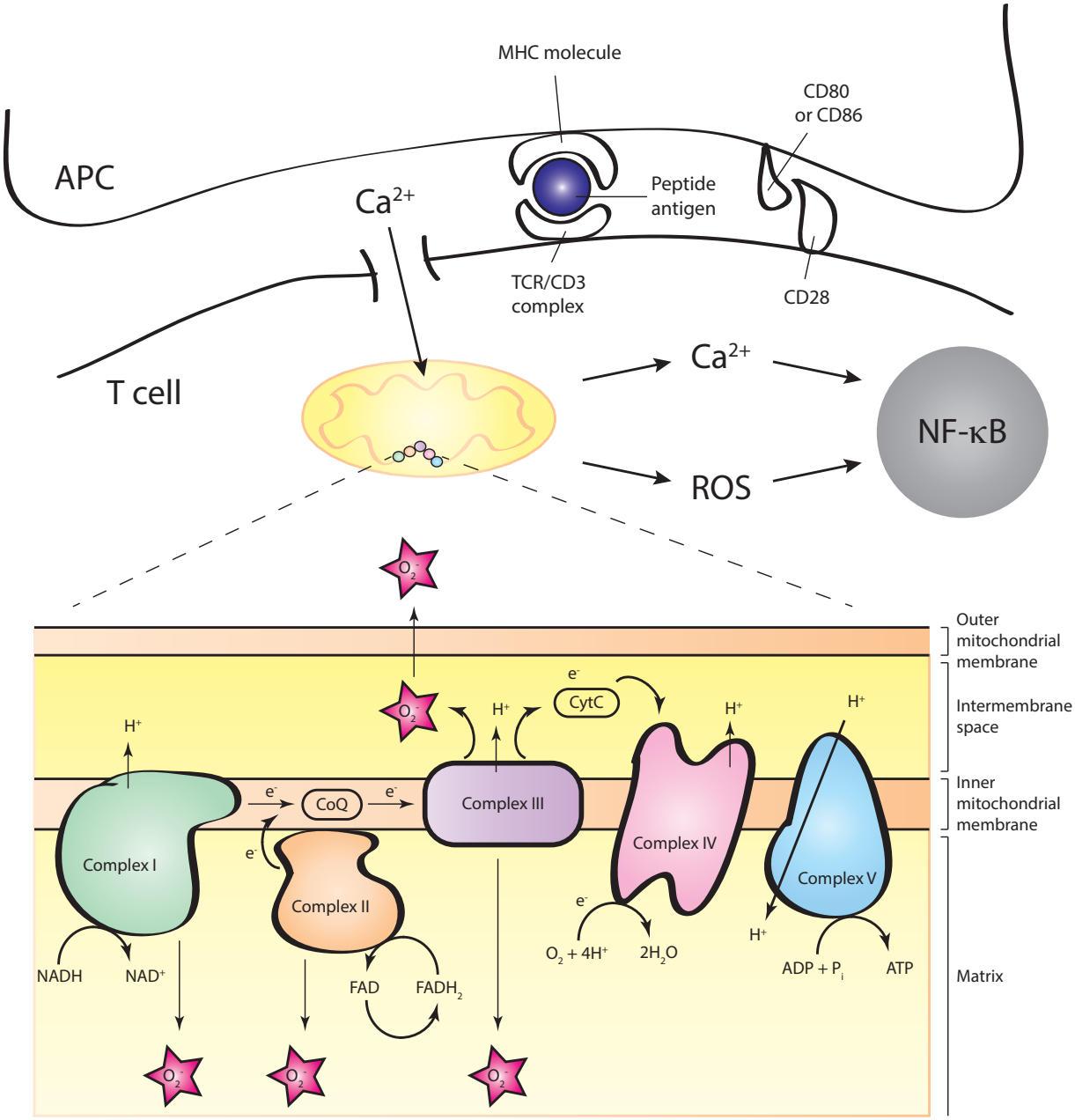
IL-12 and IL-18, two cytokines derived from antigen-presenting cells (APC; such as activated macrophages), stimulate Th1 responses and IFN-γ production (Trinchieri, 1994; Okamura et al., 1998). Although IL-12 induces Th1 differentiation directly, IL-18 acts as a co-activator rather than as an initiator for Th1 responses (Robinson et al., 1997). IL-12 alone or IL-18 alone induce only trace amounts of IFN-γ in T cells and there is a marked synergism of IL-12 and IL-18 in IFN-γ induction (Nakahira et al., 2002). IL-12 and IL-18 use different signaling pathways to activate IFN-γ production (Scheme 3). IL-12 receptor stimulation induces tyrosine phosphorylation of the Janus family kinases Jak2 and Tyk2, which results in phosphorylation of STAT4 (signal transducer and activator of transcription 4) and its translocation to the nucleus (Bacon et al., 1995). The IL-18 receptor signals through IRAK1 (IL-1 receptor-associated kinase) and TRAF6, resulting in NF-κB activation (Robinson et al., 1997). IFN-γ secreted by Th1 cells or natural killer (NK) cells has an important role in both innate and adaptive immunity. It is intimately involved in the pathogenesis of several diseases such as septic shock and systemic lupus erythematosus (Farrar and Schreiber, 1993; Boehm et al., 1997; Theofilopoulos et al., 2001).

Overall, the regulation of T cell activation, proliferation and apoptosis are fundamental for maintenance of T cell homeostasis, establishment of tolerance, and suppression of autoimmune disease. Since p21 controls T cell tolerance and protects from autoimmunity, deciphering the precise effect of p21 on the overall immune response will help to evaluate the potential of p21-related therapeutic approaches to disease.

Mitochondrial ROS regulate T cell activation

Correct activation of naïve T cells requires two signals received from the APC (Scheme 4). The first signal is delivered by binding of a specific antigen presented by major histocompatibility complex (MHC) molecules on the APC to the TCR/CD3 complex. The second signal, also referred to as a co-stimulatory signal, arises from CD28 stimulation with B7 ligand (CD80 and CD86) expressed on an APC. These signals trigger the T cell activation program that initiates rapid proliferation and differentiation to effector subsets (Th1, Th2, Th17 or Treg), as dictated by the invading organism type and cytokine signals from other inflammatory cells.

SCHEME 4 | Mitochondrial ROS in T cell activation. Naïve T cell activation requires two signals received from the antigen-presenting cell (APC): TCR/CD3 complex activation by a specific antigen presented by major histocompatibility complex (MHC) molecules on the APC, and co-stimulatory signal coming from interaction of CD28 with a B7 ligand (CD80 and CD86) expressed on an antigen-presenting cell. Activation of naïve T cells triggers Ca^{2+} influx across the plasma membrane through the opening of calcium release-activated calcium channels. Mitochondria take up Ca^{2+}, thereby reducing its local accumulation and prolonging Ca^{2+} entry. Ca^{2+} directly activates key mitochondrial enzymes, increasing mitochondrial oxidative phosphorylation and ATP production, which generates mROS. In the cytoplasm, ROS released from mitochondria together with Ca^{2+} trigger the activation of transcription factors, including NF-κB.



Activation of naïve T cells initiates immediate formation of an organized immunological synapse (IS) (Qi et al., 2001) and Ca^{2+} influx across the plasma membrane through opening of Ca^{2+} release-activated calcium (CRAC)/ Orai1 channels (Yeromin et al., 2006). Elevated Ca^{2+} levels in the cytoplasm trigger activation of transcription factors, including nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and nuclear factor of kappa light chain enhancer in B cells (NF-κB), to promote expression of proteins that drive T cell activation such as interleukin 2 (IL-2) (Smith-Garvin and Koretzky, 2009). Immune synapse formation

induces rapid translocation of mitochondria to the immediate vicinity of the IS. In fact, Ca^{2+} influx depends on coupling between mitochondria and the IS, since mitochondria uptake of Ca^{2+} reduces its local accumulation and prolongs Ca^{2+} entry, which allows efficient production of cytokines for an extended period (Quintana et al., 2007).

Whereas quiescent cells only need metabolism to support housekeeping functions, proliferating cells must produce more ATP for enhanced activity and biosynthesis. When activated, T cells therefore switch from a low rate of glycolysis and oxidation of pyruvate, to a high glycolysis rate followed by lactic acid fermentation, similar to the Warburg effect described in tumor cells (Warburg, 1956). Ca^{2+} activates key mitochondrial enzymes directly, causing an increase in tricarboxylic acid (TCA) flux (Tarasov et al., 2012), which generates NADH and $FADH_2$, electron donors for the electron transport chain. Activated T cells thus increase their mitochondrial respiration and ATP production, resulting in increased production of mitochondrial reactive oxygen species (mROS).

ROS have long been considered harmful metabolism by-products that damage cell lipids, proteins and nucleic acids (Finkel and Holbrook, 2000). Over the last decade, it has become evident that ROS are also crucial participants in cell signaling. ROS are small oxygen-containing molecules that are highly reactive due to unpaired electrons. The most common ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). Mitochondrial oxidative phosphorylation is a major source of cellular ROS (Murphy, 2009). The mitochondrial respiratory chain (which carries out oxidative phosphorylation) is composed of five multi-subunit protein complexes localized in the inner mitochondrial membrane (see Scheme 4). Electrons (e^-) are donated to complex I from NADH or to complex II from $FADH_2$, and are passed to coenzyme Q (CoQ, also known as ubiquinone), which then carries them to complex III. From complex III, electrons are passed on to cytochrome c (Cyt c) and then to complex IV, where they participate in forming water (H_2O) from hydrogen ions (H^+) and molecular oxygen (O_2). This electron movement is coupled to H^+ pumping across the inner mitochondrial membrane from the matrix to the intermembrane space, which generates an electrochemical gradient used by complex V (known as ATP synthase) to generate ATP from ADP and inorganic phosphate (P_i). During oxidative phosphorylation, approximately 1-2% of consumed oxygen is converted into superoxide when electrons leak prematurely from complex I, II and II of the electron transport chain and are transferred to molecular oxygen (Orrenius et al., 2007; Koopman et al., 2010). Superoxide can then cross the outer mitochondrial membrane via a voltage-dependent anion-selective channel, or be converted to H_2O_2 by superoxide dismutase (SOD) (Han et al., 2003). H_2O_2 can freely cross the mitochondrial membrane and either react with specific amino acids (thereby activating signaling molecules such as phosphatases, kinases or transcription factors), or can be converted to OH^- (which damages nearby molecules) or to H_2O and O_2 (by a catalase or peroxidase).

A study using the mitochondrial complex I inhibitor rotenone showed that mROS is essential for correct T cell activation and IL-2 production (Kaminski et al., 2010). This study suggested that complex I-derived mROS facilitate cytokine production by regulating AP-1 and NF-κB. In another study using mice with a targeted mutation in a complex III subunit, it was demonstrated that mROS is needed for antigen-specific T cell expansion and IL-2 production (Sena et al., 2013). The authors also

showed that Ca^{2+} influx into the mitochondria is necessary for mROS production.

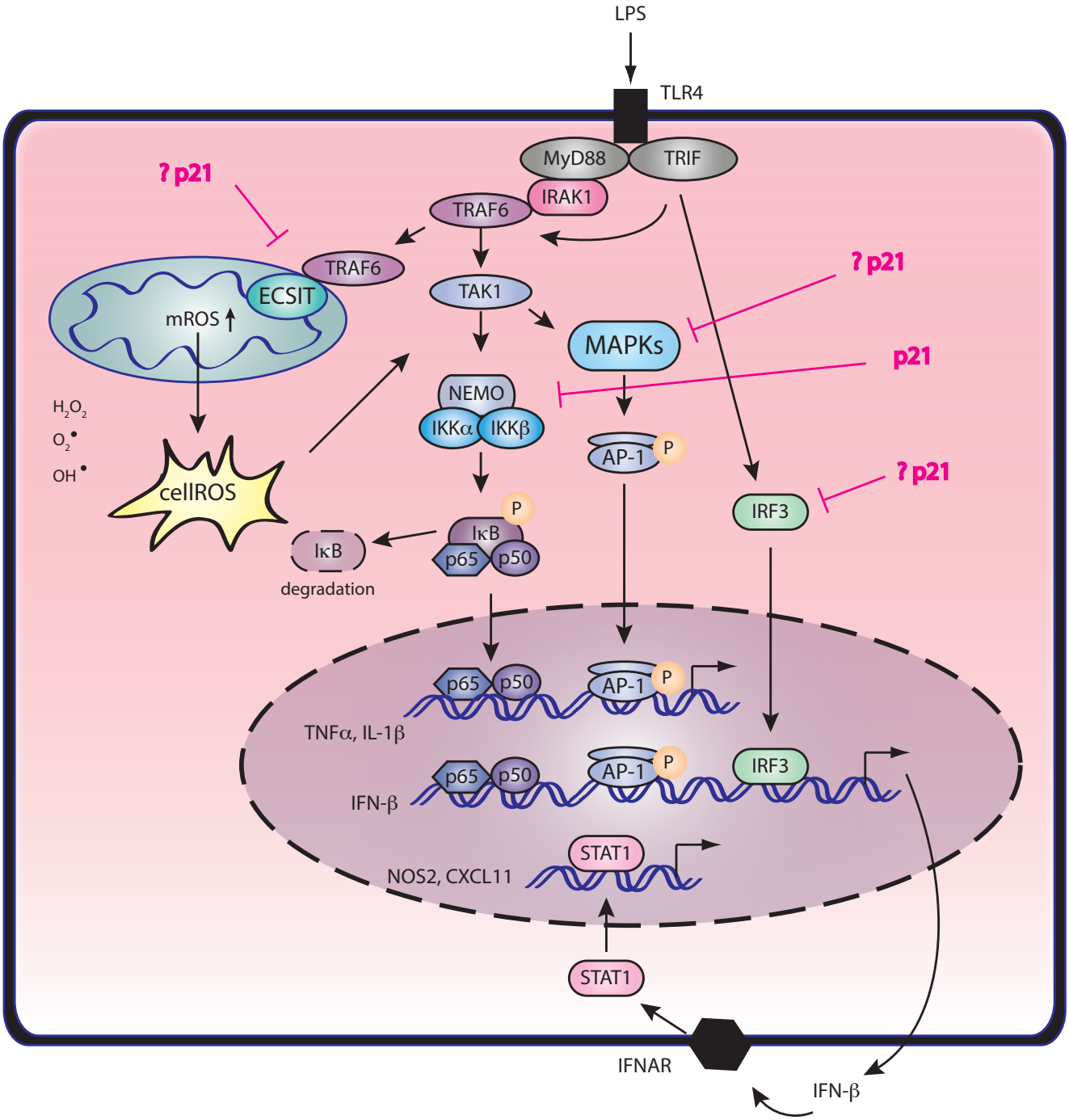
The role of mitochondria in memory T cell generation has been studied mainly in $CD8^+$ cells. $CD8^+$ memory cells show increased mitochondrial numbers and spare respiratory capacity, which relies on fatty acid oxidation to generate ATP (van der Windt and Pearce, 2012). TRAF6 (TNF receptor-associated factor 6) regulates $CD8^+$ memory cell formation by modulating fatty acid metabolism (Pearce et al., 2009). Although there is mounting evidence that mitochondrial metabolism maintains memory T cell phenotype, it is not known whether mROS participate in the signaling necessary to establish the memory T cell phenotype.

In conclusion, mitochondria play a central role in the early signaling events that control T cell activation. Increased Ca^{2+} influx maintained by the mitochondria at the immune synapse as well as mitochondrial ROS production, both induce activation of key transcription factors that regulate T cell function. Finally, the idea that mitochondrial metabolism is needed for T cell activation is further supported by observations that chronically activated T cells isolated from mouse models of lupus are dependent on mitochondrial metabolism, and peripheral blood lymphocytes from lupus patients have increased mitochondrial ROS production (Gergely et al., 2002; Wahl et al., 2010).

Macrophage activation and mitochondrial ROS

Monocytes and macrophages have essential functions in defense against pathogens and activation of adaptive immunity, as well as in initiation and resolution of inflammation (Gordon and Taylor, 2005; Lawrence and Natoli, 2011). In response to diverse microbial and environmental signals, macrophages can polarize into different phenotypes with distinct effector functions, of which two extremes are classically activated (inflammatory or M1) and alternatively activated macrophages (anti-inflammatory or M2) (Mosser and Edwards, 2008; Biswas and Mantovani, 2010; Gordon and Martinez, 2010; Mills, 2012). Macrophage plasticity is not limited to M1 and M2, but includes intermediate states with distinct polarization levels imposed by functional circumstances, some of which are described as M2a, Mb and M2c (Mantovani et al., 2004; Edwards et al., 2006). M1 macrophages are generated in response to IFN-γ (produced by T cells) or microbial LPS; they produce large amounts of proinflammatory cytokines and eliminate intracellular microorganisms and tumor cells (Mantovani et al., 2002; Sica and Mantovani, 2012). In contrast, different forms of M2 macrophages can be generated by various stimuli, such as IL-4, IL-13, IL-10, TGF-β, glucocorticoids or immune complexes; they are involved in resolution of inflammation and parasite clearance, and are also identified as resident macrophages (Mantovani et al., 2002; Gordon and Martinez, 2010; Sica and Mantovani, 2012; Satoh et al., 2013; Mills, 2012). Macrophage plasticity is a key characteristic for maintaining balance between M1 and M2 forms, as loss of optimal representation and/or appropriate recruitment can lead to harmful imbalance. Increased levels of M1 macrophages are associated with autoimmune and inflammatory diseases such as lupus and multiple sclerosis (Orme and Mohan, 2012; Shechter and Schwartz, 2013), while a predominant M2 phenotype promotes tumor growth and development of allergic asthma (Dasgupta and Keegan, 2012; Hao et al., 2012).

LPS (lipopolysaccharide), a glycolipid component of the outer membrane of Gram-negative bacteria, is a major activating agent of



SCHEME 5 | **Macrophage activation.** After binding to LPS, TLR4 induces production of proinflammatory cytokines and IFN-β in a MyD88- and TRIF- dependent manner. MyD88 recruits IRAK1, which associates with TRAF6, leading to activation of TAK1, which in turn activates two distinct pathways involving IKK complex and MAPK pathway. IKK activity results in IκB phosphorylation and its subsequent degradation, which releases NF-κB and allows its nuclear translocation where it activates inflammatory cytokines, such as TNF-α, as well as IFN-β. On the other hand, IFN-β expression can be induced by IRF3, which is activated through TRIF in an MyD88-independent pathway. Once secreted, IFN-β causes autocrine activation of the IFNAR complex, leading to STAT1 phosphorylation and subsequent induction of IFN-responsive genes (iNOS, CXCL11). Thus, IFN-β sustains M1 inflammatory response following LPS stimulation. The role of p21 in negative regulation NF-κB through IKK has been established (Trakala et al., 2009). However, the mechanism how p21 controls macrophage activation remains unexplained.

macrophages that acts through TLR4 (Toll-like receptor 4) (Scheme 5). After binding to LPS, TLR4 induces production of proinflammatory cytokines and IFN-β in a MyD88 (myeloid differentiation primary-response protein 88) and TRIF (Toll/interleukin-1 receptor-domain-

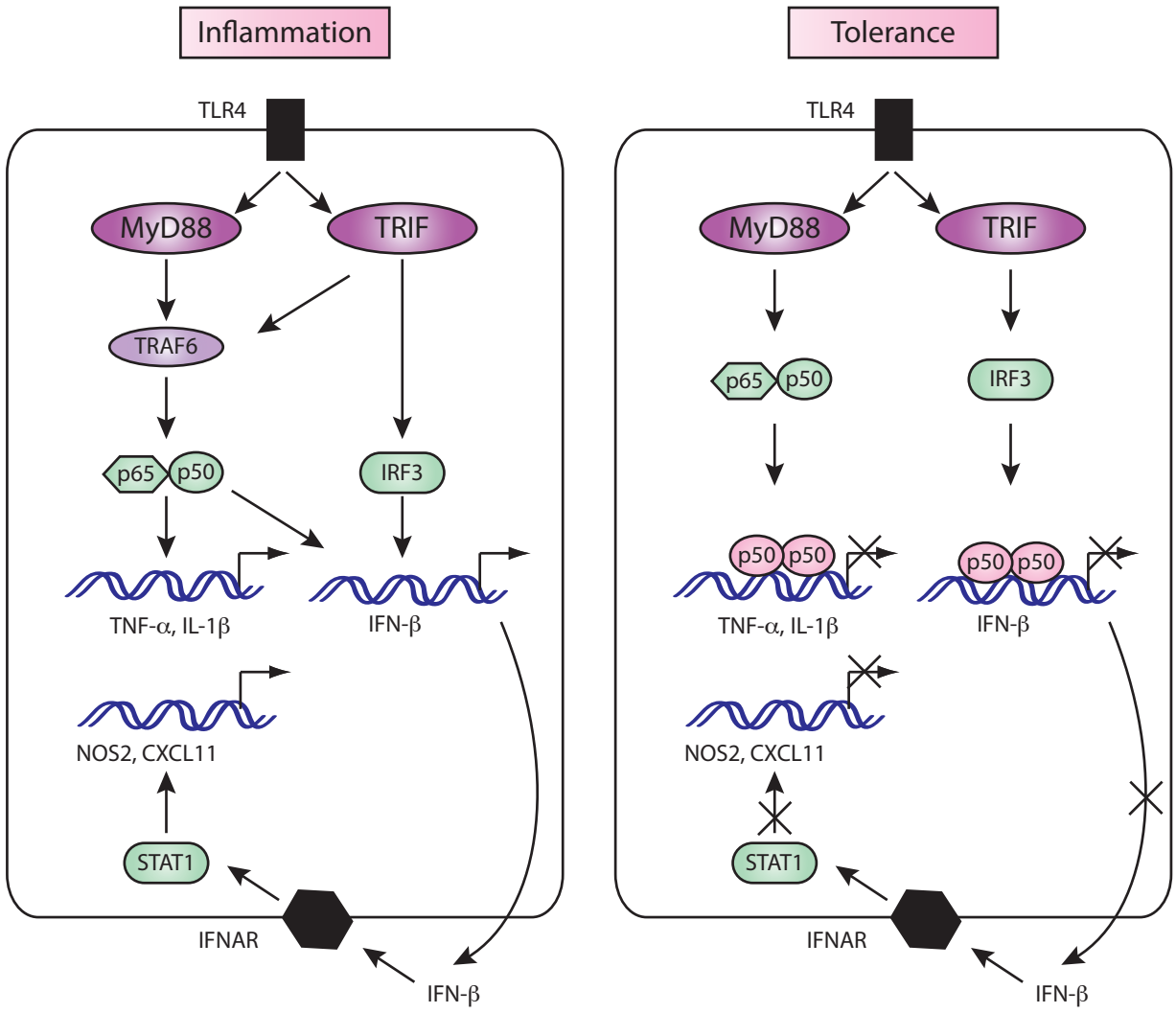
containing adaptor protein inducing IFN-β) -dependent manner. MyD88 recruits IRAK1 (IL-1 receptor-associated kinase 1), which associates with TRAF6 (TNF receptor-associated factor 6), leading to activation of TAK1, which in turn activates two distinct pathways involving the IKK complex (inhibitor of κB kinase) and the MAPK (mitogen-activated protein kinase) pathway (Kawai and Akira, 2007). IKK activity results in IκB phosphorylation and subsequent degradation; this releases NF-κB and allows its translocation to the nucleus where it activates inflammatory cytokines such as TNF-α as well as IFN-β. IFN-β expression can also be induced by IRF3 (IFN-regulatory factor 3), which is activated through TRIF in an MyD88-independent pathway (Kawai and Akira, 2010). Once secreted, IFN-β causes autocrine activation of the IFNAR (IFN-α/β receptor) complex, leading to phosphorylation of STAT1 (signal transducer and activator of transcription 1) and subsequent induction of IFN-responsive genes (iNOS, CXCL11). IFN-β thus sustains the M1 inflammatory response following LPS stimulation.

ROS are essential components of macrophage phagocytic activity and are needed for destruction of intracellular bacteria. It is thought that macrophages generate ROS primarily via the phagosomal NADPH oxidase machinery (Lambeth, 2004). Several studies suggest that ROS generated during mitochondrial oxidative phosphorylation also contribute to macrophage bactericidal activity, implicating mitochondria as signaling organelles in innate immunity (Arsenijevic et al., 2000; Chandel et al., 2000, 2001; Rousset et al., 2006). After TLR4 activation, TRAF6 can translocate to mitochondria, where it interacts with proteins involved in the mitochondrial respiratory chain, leading to increased mitochondrial and cellular ROS generation (West et al., 2011a). Patients with tumor necrosis factor receptor-associated periodic syndrome (TRAPS) have heightened responsiveness to LPS due to increased production of mitochondrial ROS (mROS) (Bulua et al., 2011). There is considerable evidence that mROS induced by TLR4 activation acts as an important component of this signaling pathway through redox-sensitive activation of the IKK complex and NF-κB, leading to pro-inflammatory cytokine production (Bai et al., 2005; Gloire et al., 2006; West et al., 2011b).

In addition to lymphocyte abnormalities, aberrations in monocyte/macrophage functions have been recognized increasingly to contribute to induction and development of autoimmunity (Li et al., 2010). TLR activation and the production of type I interferons (IFN-α/β) were recently implicated in the onset of systemic lupus erythematosus (SLE) pathogenesis (Theofilopoulos, 2012). IFN-β can be produced by several cell types including macrophages, in response to TLR engagement during viral or bacterial infection, or to endogenous stimuli such as self-nucleic acid-containing immune complexes and necrotic debris (Elkon and Stone, 2011). IFN-β release primes plasmacytoid dendritic cells to produce IFN-α, which is present in large amounts in lupus patient serum (Hooks et al., 1979). Interferons are thought to have a pathogenic role in autoimmunity and in particular in SLE, supported by the finding of IFN-inducible genes in the peripheral blood gene expression profile in individuals with SLE (Rönnblom et al., 1991; Baechler et al., 2003; Santiago-Raber et al., 2003). Moreover, serum levels of chemokines related to IFN activity are elevated in SLE patients (Kong et al., 2009). In humans, IFN-γ administration can induce SLE or autoantibody production (Graninger et al., 1991). Conversely, administration of anti-IFN-γ or -IFNα/β receptor (IFNAR) antibodies decreases the levels of IFN-inducible genes in patient peripheral blood and ameliorates the disease in mice predisposed to lupus-like

disease (Santiago-Raber et al., 2003; Baccala et al., 2012; Welcher et al., 2015).

Type I interferons have long been known as potent antiviral molecules, and a critical role has been elucidated for IFN in LPS-induced endotoxemia. IFN-β knockout and IFNAR-deficient mice are more resistant to LPS-induced shock than WT mice (Karaghiosoff et al., 2003; Mahieu et al., 2006). In fact, IFNs mediate TNF-α induction during septic shock (Huys et al., 2009). Blocking IFN as well as proinflammatory cytokines appears to be a possible therapeutic strategy in experimental endotoxemia and in sepsis patients (Mahieu and Libert, 2007). In animal models of sepsis, neutralization of proinflammatory cytokines, such as TNF-α and IL-1β, severely reduces mortality (Beutler et al., 1985), although clinical trials did not show such success in patients (Fisher et al., 1996). In contrast to animals, which die of septic shock within 72 hours, patients die much later due to opportunistic infections and inability to produce proinflammatory cytokines in response to LPS. IFN-γ treatment was therefore proposed as an immunostimulatory therapy to restore monocyte activation in patients at the hypoinflammatory stage (Döcke et al., 1997).



SCHEME 6 | **TLR4 signaling during LPS tolerance.** This figure summarizes the importance of inhibitory p50/p50 NF-κB homodimer for shutting down proinflammatory gene expression, and the possible involvement of IFN-β in promoting sustained proinflammatory activation which could interfere with LPS tolerance.

Macrophage reprogramming during LPS tolerance and sepsis

After long or repeated exposure to LPS, macrophages undergo functional reprogramming; they downregulate their proinflammatory M1 profile and acquire an M2 phenotype (Porta et al., 2009; Pena et al., 2011) (Scheme 6). These cells, referred to as LPS- or endotoxin-tolerant macrophages, are hyporesponsive to subsequent LPS challenge and no longer produce proinflammatory mediators such as TNF-α, IFN-β and iNOS (inducible nitric oxide synthase) (Foster et al., 2007; Piao et al., 2009). Consistent with their M2 phenotype, LPS-tolerant macrophages upregulate prototypical M2 genes such as arginase I, IL-10 and Th-2-associated chemokines like CCL2 (Porta et al., 2009; Cubillos-Zapata et al., 2014). These M2 cells might nevertheless show important features that render them distinct from typical M2 macrophages (Pena et al., 2011).

The refractory state of human monocytes after induction of endotoxin tolerance is also known as hyporesponsiveness or an immunosuppressive state; it has raised scientific interest, as a similar state of monocyte deregulation is observed in sepsis patients (Cavaillon and Adib-Conquy, 2006; Pena et al., 2014; Shalova et al., 2015). Sepsis is a complex condition characterized by large amounts of circulating proinflammatory cytokines, caused by a decontrolled innate response following systemic bacterial infection (Angus and van der Poll, 2013). Inflammatory cells subsequently enter a temporary state of hyporesponsiveness and become tolerant to further endotoxin challenge. Monocytes isolated from sepsis patients are thus characterized by low proinflammatory cytokine profiles when they are stimulated *ex vivo* with LPS (Escoll et al., 2003). Monocyte hyporesponsiveness in sepsis does not correspond to simple downregulation of proinflammatory cytokine production, but rather to a reprogrammed state with alternative functional activity (Escoll et al., 2003; Cavaillon and Adib-Conquy, 2006; Shalova et al., 2015). Immunosuppression in sepsis was first considered a compensatory mechanism that regulates hyperinflammation to alleviate the deleterious effects of septic shock (Biswas and Lopez-Collazo, 2009; López-Collazo and Del Fresno, 2013). This anti-inflammatory response correlates with sepsis progression and death, however, and might increase the risk of secondary infection (Angus and van der Poll, 2013) or lead to influx of other inflammatory cells (Ariga et al., 2014; Pena et al., 2014). In mice, septic shock can be induced by a single LPS delivery, which corresponds to an initial proinflammatory response in sepsis, whereas endotoxin tolerance induced by LPS priming and rechallenge drives macrophage immunosuppression (Berg et al., 1995; Wysocka et al., 2001) and can be used as a model to study hyporesponsiveness in sepsis (López-Collazo and Del Fresno, 2013).

One of the major characteristics of endotoxin tolerance is the downregulation of proinflammatory cytokines such as TNF-α and IFN-β (Biswas and Tergaonkar, 2007). TNF-α regulation depends on NF-κB activation through TLR4 stimulation (Scheme 6). Although diverse pathways have been considered for IFN-β regulation (Toshchakov et al., 2002; Akira and Takeda, 2004), data from $p50^{-/-}$ mouse macrophages after LPS activation have also linked IFN-β to the NF-κB pathway and p50-regulating activity (Porta et al., 2009). In addition, as it has an essential function during Gram-negative bacterial infection in humans, a role for IFN-β in the pathology of human sepsis is proposed (Dejager et al., 2013).

The mechanism that regulates macrophage reprogramming from M1 to M2 during endotoxin tolerance remains largely undefined.

Nevertheless, overexpression of certain negative regulators of the TLR4 pathway, including IRAK-M (interleukin-1 receptor-associated kinase-M), A20 and SHIP (src homology 2-containing inositol-5'-phosphatase) is associated with macrophage unresponsiveness (Kobayashi et al., 2002; Sly et al., 2004; Biswas and Lopez-Collazo, 2009; Xiong and Medvedev, 2011). The IRAK-M inducer HIF1α (hypoxia inducible factor-1α) was recently identified as a mediator of endotoxin tolerance in sepsis (Shalova et al., 2015). Accumulation of the p50 NF-κB subunit and predominance of p50/p50 over p65/p50 NF-κB levels are linked to macrophage hyporesponsiveness (Ziegler-Heitbrock, 2001; Adib-Conquy et al., 2000). p50 lacks a transcription activation domain and, when bound as a homodimer to gene promoters, it blocks binding of active p65/p50 NF-κB and thus inhibits gene expression (Carmody and Chen, 2007; Hayden and Ghosh, 2008). p50 deficiency leads to defective macrophage tolerance and M1 to M2 polarization (Porta et al., 2009). NF-κB regulation is therefore a key event in orchestrating M1 *vs.* M2 responses in LPS-tolerant macrophages, while the mechanism that regulates this NF-κB-dependent plasticity during endotoxin tolerance remains to be defined.

OBJECTIVES

As a small molecule able to participate in a number of specific protein-protein interactions, p21 plays a critical role in the regulation of many biological functions, such as cell cycle, apoptosis, cell differentiation and senescence, oxidative stress, and gene expression (Dotto, 2000). Ever since the initial discovery that p21-deficient mice develop lupus-like autoimmune disease (Balomenos et al., 2000), our group has focused on deciphering the role of p21 in regulation of immune responses. It was shown that in absence of p21, T cells hyperproliferate, leading to break of tolerance (Balomenos et al., 2000). p21 does not affect naïve T cells, but exclusively regulates the proliferation of effector/memory T cells that survive AICD (Arias et al., 2007). We recently showed that this p21 property has a therapeutic effect, since transgenic overexpression of p21 in T cells of lupus-prone B6/*lpr* mice reduces proliferation and activation of autoreactive T cells and ameliorates the disease (Daszkiewicz et al., 2015). Several reports, including the data from our group, also showed a role for p21 in regulation of innate immunity. In the absence of p21, macrophages have increased NF-κB activity and proinflammatory cytokine production in response to LPS, making $p21^{-/-}$ mice extremely sensitive to septic shock (Trakala et al., 2009; Scatizzi et al., 2009). These reports clearly link p21 with regulation of both innate and adaptive immune responses. The mechanism by which p21 regulates T cells and macrophages, as well as its impact in human patients, nonetheless remained unexplained.

We therefore established the following objectives for the present study:

1. To investigate whether the role of p21 in controlling effector/memory $CD4^+$ T cell activation is physiologically relevant for autoimmunity.
2. To study whether p21 controls IFN-γ production directly in effector/memory $CD4^+$ T cells.
3. To obtain mechanistic insight into how p21 controls effector/memory $CD4^+$ T cell activation and IFN-γ production.
4. To analyze the role of p21 in TLR4-dependent macrophage activation.
5. To determine whether p21, as a negative regulator of TLR4 activation, affects macrophage reprogramming during LPS tolerance.
6. To evaluate the mechanism by which p21 regulates macrophage reprogramming during LPS tolerance.
7. To verify whether our findings on the role of p21 in macrophage reprogramming during LPS tolerance are relevant for human sepsis.

MATERILAS AND METHODS

Animals

Mice were kept in the barrier zone of our animal facility to avoid contact with pathogens. Six to eight weeks old female mice were used for all experiments. C57BL/6 (B6, or WT) mice were from Harlan Interfauna Ibérica; $p21^{-/-}$ mice were obtained as described previously (Trakala et al., 2009). C57BL/6-*lpr* (B6/*lpr*) mice were from Jackson Laboratories. In C57BL/6-*lpr*-p21tg (B6/*lpr*-p21tg) mice, generated from C57BL/6-*lpr* and C57BL/6-p21tg (B6-p21tg) mice, transgenic expression was restricted to T cells by the proximal Lck promoter (Fotedar et al., 1999). C57BL/6-*lpr*-$p21^{-/-}$ ($p21^{-/-}$/*lpr*) mice were generated from C57BL/6-*lpr* and C57BL/6-$p21^{-/-}$ mice.

Allergic asthma

To induce allergic asthma, five WT and $p21^{-/-}$ mice were sensitized by two i.p. injections of 10 μg OVA in 200 μl alum at day 0 and day 11, whereas control mice were sham-sensitized only with alum. Then from day 19 to day 24 mice were challenged each day for 20 min with aerosolized 1% OVA in PBS inhalation and sacrificed 24 h after the last treatment for histological analysis of lung inflammation. Control mice were sham-sensitized only with alum and aerosol challenged with PBS. Blood was obtained by cardiac puncture and OVA-specific IgE in serum was determined by sandwich ELISA (Cayman Chemical). Lungs were fixed in paraformaldehide and tissue sections were stained with hematoxylin and eosin for the evaluation of the inflammatory infiltrate.

Induction of LPS tolerance *in vivo*

Mice were injected i.p. with 30 μg of LPS (from E. coli 0127:B8), followed 16 h later by an i.v. injection of 150 μg of LPS. Survival was monitored for 72 h. At 2 h post-LPS rechallenge, peritoneal exudates were harvested and blood was collected by cardiac puncture. For the IFNAR blocking experiments, two groups of $p21^{-/-}$ mice (n = 9 per group) were injected i.p. with 300 μg of IFNAR1 monoclonal antibody (MAR1-5A3, BioXCell), or a control IgG antibody (BioXCell) 2 h before the LPS rechallenge.

Serological analyisis

IFN-γ levels in T cell-free supernatants were determined by FlowMetrix system (Luminex, Austin, TX).

We tested mouse blood serum and cell-free culture supernatants by ELISA for TNF-α (PeproTech) and IFN-β (PBL Assay Science) levels. The cytokine levels in the culture supernatants from the human samples were determined using the cytometric bead array (CBA) Flex Set (BD Biosciences) following the manufacturer's protocol. The data were collected and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences).

Flow cytometry

To determine memory phenotype and activation, T cells were stained with anti-CD4-PeCy7, -CD44-APC, -CD62L-PE and -CD69-FITC (Beckman Coulter) antibodies.

Peritoneal exudates were stained with anti-F4/80-APC (eBioscience), -CD11b-PE-

Cy7 (BioLegend), -I-A^b-FITC (Thermo Fisher Scientific) and -CD40-PE (BioLegend) and analyzed on a Gallios cytometer (Beckman Coulter). The data were analyzed with FlowJo software (Tristar).

Intracellular cytokine staining

For intracellular cytokine detection, lymph node T cells were stimulated (4 h) in medium with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin (2 μg/ml). Alternatively, for *in vitro* experiments after primary activation and IL-2 expansion, cells were stimulated with IL-12 plus IL-18 for 24 h. Brefeldin A (1 ×, BioLegend) was added during the last 3 h of stimulation. Cells were stained with violet LIVE/DEAD stain kit (Invitrogen) to exclude dead cells. After surface marker staining with anti-CD4-PeCy7, -CD44-APC, -CD62L-PE, cells were washed with PBS, fixed and permeabilized with Citofix/Citoperm kit (BD Biosciences) and blocked with 1 μl of Fc-receptor blocking antibody (Beckman Coulter) for 15 min at 4°C. Cells were stained intracellularly with anti-IFN-γ-FITC (1/100 dilution, eBioscience) and analyzed on a Gallios cytometer (Beckman Coulter).

In LPS tolerance experiments, WT and $p21^{-/-}$ mice were i.v. injected with Brefeldin A (500 ml of 1 × Brefeldin A in PBS; BioLegend) 30 min post-LPS rechallenge. After 3 h, peritoneal exudates were harvested and stained with violet LIVE/DEAD stain kit (Invitrogen) to exclude dead cells. After surface marker staining with anti-CD11bPECy7 (BioLegend) and -F4/80-APC (eBioscience), cells were washed with PBS, fixed and permeabilized with Citofix/Citoperm kit (BD Biosciences) and blocked with 1 μl of Fc-receptor blocking antibody (Beckman Coulter) for 15 min at 4°C. Cells were stained intracellularly with anti-TNF-α-PE (1/100 dilution, eBioscience) and analyzed on a Gallios cytometer (Beckman Coulter).

Cell culture

Mouse $CD4^+$ spleen T cells were purified by Negative Isolation Kit (Dynal Biotech); >85%pure $CD4^+$ T cells were obtained. Purified naïve T cells (10^6/ml) were stimulated *in vitro* with concavalin A (ConA, 1.5 μg/ml, Sigma) in medium containing 20 ng/ml human recombinant interleukin 2 (IL-2, PeproTech), or in medium conditioned with anti-CD28 (PharMingen, 1 μg/ml) in wells coated with anti-CD3 (PharMingen, 1 μg/ml in PBS). After 1 day, cells were washed and cultured in medium with 20 ng/ml IL-2 for 6 days, followed by restimulation with IL-2 in combination with either ConA, or anti-CD3/CD28, or IL-12 (10 ng/ml) plus IL-18 (10 ng/ml).

For macrophage cultures, peritoneal exudate cells (PEC) were harvested from B6/*lpr* mice that were injected i.p. with 1 ml of 3% thioglycollate medium (Difco) 4 days before isolation. PEC were cultured for 2 h in RPMI 1640 without FBS and non-adherent cells were removed by washing with PBS; adherent macrophages were cultured overnight in complete RPMI 1640 with 10% FBS. Cells were incubated for 20 h in the presence of 1:2 diluted cell-free culture supernatants from B6/*lpr* or B6/*lpr*-p21tg (collected 24 h after second ConA stimulation). Control cells were cultured in medium alone or stimulated for 20 h with IFN-γ (100 or 50 U/ml, Sigma).

To induce tolerance, adherent peritoneal macrophages from WT and $p21^{-/-}$ mice were treated with LPS (100 ng/ml; E. coli 0127:B8; Sigma) for 20 h, washed with PBS, maintained in medium for 2 h and then restimulated with LPS (100 ng/ml) for 4 h (12). Control cells were tolerized with LPS (20 h), washed and maintained in the medium for 6 h, without LPS rechallenge. To induce LPS activation, cells were incubated in medium for 20 h, washed, left in medium for 2 h and stimulated with LPS

for 4 h. Unstimulated cells were maintained in medium throughout the entire experiment. IFN-β depletion assays were performed by incubating with anti-IFN-β antibody (7F-D3; Yamasa Corporation), or isotype control (Rat IgG1, Antigenix America) at a concentration of 6.7 μg/ml.

The monocytes were obtained from peripheral blood and purity was tested as previously described (Cubillos-Zapata et al., 2014). To establish endotoxin tolerance, monocytes were tolerized with 10 ng/ml LPS (16 h), washed with PBS and rechallenged with 10 ng/ml LPS for indicated time periods. The control cells were not tolerized and/or rechallenged with LPS.

Bone marrow differentiation

To obtain bone marrow-derived macrophages (BMDM), bone marrow was collected from WT and $p21^{-/-}$ mice and cultured in Petri dishes for 7 days in DMEM containing 10 % FBS plus 30% L929 conditioned medium. Medium was replenished on day 4 of the culture. On day 7, >95% pure $CD11b^{+}F4/80^{+}$ macrophages were used for experiments.

To generate dendritic cells (DC), bone marrow progenitor cells from B6 mice were cultured with GM-CSF (20 ng/ml) obtained from supernatant of the melanoma tumor D5-G6 cell line. Medium was replenished on day 4 of the culture. At day 6, slightly adherent and non-adherent cells were collected with PBS/3mM EDTA, re-plated and incubated in the presence of GM-CSF (20 ng/ml) and IL-4 (1 ng/ml) for 24 h. Next, DC were stimulated with 1 μg/ml LPS (O26:B6, Sigma) and 1 mM OVA peptide (323-339) for 20 h. Non-adherent cells were characterized by flow cytometry and >60% of $CD11c^{+}MHCII^{+}CD86^{+}$ dendritic cells were obtained.

OT-II $CD4^{+}$ T cell stimulation

Purified naïve OT-II and $p21^{-/-}$ OT-II $CD4^{+}$ T cells were stimulated with specific OVA peptide (Robertson et al., 2000) presented by dendritic cells (DCs) obtained from WT (B6) mice (T cell:DC cell ratio 10:1). After 24 h of primary stimulation, T cells were expanded in IL-2 for 6 days and restimulated with OVA-loaded DCs for indicated times.

ROS measurements

Cells were incubated with MitoSOX Red (to measure mROS superoxide) or CM- H_2DCFDA (to measure total cellular H_2O_2) (Invitrogen) at 5 μM final concentration in complete medium for 30 min at 37°C. Mitochondrial ROS inhibitor DPI (5 μM, Sigma) was added to cell culture 1 h before the stimulus. Median fluorescence intensity was measured by flow cytometry. To control for baseline fluorescence, samples from each experiment were left unstimulated but stained according to the above procedure. Relative fluorescence was calculated by dividing stimulated by unstimulated values. Error bars were generated by calculating standard error of the mean from triplicate samples. All ROS experiments shown are representative of three independent experiments.

Cell cycle analysis

To analyze cell cycle, 1×10^6 cells were permeabilized with detergent, stained with PI according to the manufacturer's instructions (DNA-Prep Reagent Kit, Beckman Coulter) for 30 min at 37°C, and analyzedby flow cytometry.

Real-Time PCR

Macrophage cultures were washed with ice-cold PBS and total RNA was extracted with TRIzol (Sigma) according to the manufacturer's

instructions. Reverse transcription from 1 μg of RNA was performed using the cDNA kit (Appled Biosystems). Real-time PCR was performed using EvaGreen Master Mix (Solis BioDyne) and detected by the ABI PRISM 7900HT (Applied Biosystems). Data were processed using SDS 2.4 software (Applied Biosystems). Results were normalized to the expression of the β-actin and presented as the fold induction with respect to the control cell population. Due to high IL-10 mRNA levels in unstimulated cells, results of IL-10 expression were normalized to unstimulated cells of each genotype to calculate the amplitude of the transcription response ("dynamic range") (Karaghiosoff et al., 2003) after stimulation. The products were amplified using primers for p21, 5'-GCA GAT CCA CAG CGA TAT CC-3' (forward) and 5'-CAA CTG CTC ACT GTC CAC GG-3' (reverse); arginase I, 5'-CAC TCC CCT GAC AAC CAG CT-3' (forward) and 5'-AAG GAC ACA GGT TGC CCA TG-3' (reverse); YM1, 5'-ACT TTG ATG GCC TCA ACC TG-3' (forward) and 5'-AAT GAT TCC TGC TCC TGT GG-3' (reverse); CCL17, 5'-GGC CTT GGG TTT TCA CCA-3' (forward) and 5'-CAG GGA TGC CAT CGT GTT TC-3' (reverse); TNF-α 5'-CTG TAG CCC ACG TCG TAG C-3' (forward) and 5'-TTG AGA TCC ATG CCG TTG-3' (reverse); IL-1β, 5'-TGG TGT GTG ACG TTC CCA TT-3' (forward) and 5'-CAG CAC GAG GCT TTT TTG TTG-3' (reverse); IFN-β, 5'-TCA GAA TGA GTG GTG GTT GC-3' (forward) and 5'-GAC CTT TCA AAT GCA GTA GAT TCA-3' (reverse); iNOS, 5'-GCT GTG CTC CAT AGT TTC CAG-3' (forward) and 5'-GGA CCA GCC AAA TCC AGT C-3' (reverse); CXCL11, 5'-AAA GAC AGC GCC CCT-3' (forward) and 5'-GGC TGC TGA GAT GAA CAG GAA-3' (reverse); IL-10, 5'-CGG GAA GAC AAT AAC TG-3' (forward) and 5'-CAT TTC CGA TAA GGC TTG G-3' (reverse); IRAK-M, 5'-TGA GCA ACG GGA CGC TTT-3' (forward) and 5'-GAT TCG AAC GTG CCA GGA A-3' (reverse); β-actin, 5'-GGC TGT ATT CCC CTC CAT CG-3' (forward) and 5'-CCA GTT GGT AAC AAT GCC ATG T-3' (reverse). All primers were synthesized, desalted, and purified by Sigma.

For human samples, RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics). The cDNA was obtained by reverse transcription of 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed using the QuantiMix Easy SYG kit from Biotools. Results were normalized to the expression of β-actin, and the cDNA copy number for each gene was determined using a 7-point standard curve, as described previously (Del Fresno et al., 2005). The products were amplified using primers for TNF-α, 5'-GCC TCT TCT CCT TCC TGA TCG T-3' (forward) and 5'-CTC GGC AAA GTC GAG ATA GTC G-3' (reverse); CCL2, 5'-GAT CTC AGT GCA GAG GCT CG-3' (forward) and 5'-ATT CTT GGG TTG TGG AGT GAG TGT TCA-3' (reverse); p21, 5'-GAG GCC GGG ATG AGT TGG GAG GAG-3' (forward) and 5'-CAG CCG GCG TTT GGA GTG GTA GAA-3' (reverse); IFN-β, 5'-CCT GGC TAA TGT CTA TCA TCA-3' (forward) and 5'-GCA GTA CAT TAG CCA TCA GTC-3' (reverse); β-actin, 5'-AAG AGC TAC GAG CTG CCT GAC G-3' (forward) and 5'-TCC ACA TCT GCT GGA AGG TGG-3' (reverse). The primers were synthesized, desalted and purified by Bonsai Biotech.

Western blot

Cultured T cells were washed with ice-cold PBS and lysed in a buffer containing 0.2% Nonidet P-40, 10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 1 × protease and phosphatase inhibitor cocktail (Roche), for 20 min at 4°C. The lysates were centrifuged at 14,000 × *g* for 15 min; the supernatants were measured using the Bio-Rad protein assay and resolved in 12% SDS-PAGE (20 μg protein per

lane). Proteins were next transferred into a nitrocellulose membrane (75 min at 300 mA) and immunoblotted using antibodies against phospho-STAT4 (Cell Signaling), p21 (Santa Cruz Biotechnology) and actin (Sigma). HRP-conjugated secondary antibodies (Dako) were used at 1:2,000 dilution for 1 h at room temperature. Blots were visualized using Western Lightning Plus-ECL (PerkinElmer).

Macrophage lysates (20 μg protein) were analyzed using antibodies against p21, p50, p65 (Santa Cruz Biotechnology), phospho-STAT1 (Y701), phospho ERK, IκBα, phospho IRF3 (Cell Signaling), iNOS (Abcam), JNK (Invitrogen), actin (Sigma).

EMSA

NF-κB double stranded consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were purchased from Promega; double stranded oligonucleotides corresponding to the PRDII domain of the IFN-β promoter (5'-GGG AAA TTC CGG GAA ATT CC-3') and oligonucleotides with mutant NF-κB sites (5'-act AAA TTC CAC TAA ATT CC-3') were purchased from Sigma. They were end-labeled with [g^{32}P]ATP (PerkinElmer) using the T4 polynucleotide kinase (Promega). Binding reactions were prepared using 5 μg of nuclear extract in a 25 μl reaction volume containing 0.5 ng labeled oligonucleotide probe, 10 mM HEPES, 1 mM $MgCl_2$, 35 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 μg bovine serum albumin and 1.5 μg poly[d(I-C)] at room temperature for 30 min. For supershift analysis, 1 μg of antibodies against p50 (D-17) or p65 (F-6) NF-κB (Santa Cruz) were added to the reaction mixture at room temperature for 20 min prior to the addition of radiolabelled probes. Binding reactions were resolved on a 4% non-denaturing polyacrylamide gel at 300V for 1.5 h at 4°C in 0.5 × TBE. Gels were subsequently dried and exposed to X-ray film at -80°C. Densitometry was performed using Quantity One 4.6.6 software (Bio-Rad). Alternatively, gels were exposed to a phosphor screen and visualized on a Personal Molecular Imager (Bio-Rad) and similar data were obtained.

Immunoprecipitation and CDK2 kinase assay

For immunoprecipitatin and CDK2 kinase assay (Trakala et al., 2009), cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). Protein lysates (200 μg) were mixed overnight at 4° C with 2 μg anti-CDK2 (M2, Santa Cruz) and incubated with pre-blocked protein G-Sepharose beads (25 μl, Invitrogen) for additional 2 h. Anti-CDK2 immunoprecipitates were then incubated with 20 μl kinase buffer (20 mM Tric-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, protease inhibitor cocktail and phosphatase inhibitor cocktail), supplemented with 5 μg histone H1 (Roche), 0.5 mM ATP (Cell Signaling) and 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Perkin Elmer; 30 min at 30° C). Phosphorylated histone H1 was resolved by gel electrophoresis.

Patients

We included 7 subjects with sepsis secondary to a urinary tract infection (clinical details in Table I) who were admitted to the Department of Internal Medicine Service at La Paz Hospital (Madrid, Spain). The patients met the consensus conference definition of sepsis (American College of Chest Physicians/ Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis., 1992) and sepsis was confirmed by blood cultures positive for *Escherichia coli*. Peripheral blood was collected

within 24 h of sepsis confirmation. Exclusion criteria included presence of malignancy or chronic inflammatory diseases, treatment with steroids or immunosuppressive drugs during the last month, hepatic failure (serum aspartate aminotransferase and/or alanine aminotransferase > 100 IU/L; prothrombin time < 60%; total bilirubin < 60 mmol/L), renal insufficiency (plasma creatinine > 200 mmol/L), HIV/AIDS, hepatitis B or C, pregnancy and age > 80 years. Blood collected from healthy volunteers served as controls.

Small interfering RNA

p21 (ID 1531) and the control small interfering RNAs (siRNAs) were designed and synthesized by Life Technologies. Human monocytes were transfected with siRNAs using the Amaxa Nucleofector system (Amaxa Biosystems). Briefly, 1.5 × 10^6 monocytes were nucleofected with 30 μM siRNA mixed with 100 μl nucleofection solution, according to the manufactrer's instructions. The cells were then immediately transferred to a six-well culture plate (Costair) in a final volume of 2 ml prewarmed RPMI 1640 media supplemented with 10% heat-inactivated FBS (Invitrogen). The nucleofected cells were cultured at 37°C with 5% CO_2 for 1 h before the assays.

Statistical analysis

Statistical significance was determined by one-way ANOVA, two-way ANOVA (with Bonferroni correction), unpaired two-tailed Student's *t* test or Mann-Whitney *U* test, as indicated. Survival curves were generated using Kaplan-Meier method, and compared using log-rank test. The correlation analysis was assessed using Pearson correlation coefficient. All statistical analyses were conducted using Prism 6.0 software (GraphPad). **p*<0.05; ***p*<0.01; ****p*<0.001.

Study approval

All animal experiments were designed in compliance with European Union directives and guidelines, and were approved by the Centro Nacional de Biotecnología Ethics Committee. We obtained informed consent from all participants. The study was performed in accordance with the Helsinki Declaration of 2000 and was approved by the "La Paz Hospital" Ethics Committee.

RESULTS

Chapter 1: p21 regulates effector/memory CD4$^+$ T cell activity and IFN-γ production through mitochondrial reactive oxygen species

Studies of p21$^{-/-}$ mice suggested that p21 controls the proliferation of TCR-stimulated but not naïve T cells, leading to development of lupus-like autoimmune disease (Balomenos et al., 2000; Arias et al., 2007). These studies show that p21 does not affect Fas/Fas ligand (FasL)-mediated apoptosis, and suggest that p21 regulates the proliferation of apoptosis-resistant effector/memory T cells. p21 deficiency in autoimmunity-prone mice leads to severe lupus-like disease and death (Bygrave et al., 2004; Balomenos et al., 2000; Salvador et al., 2002). To provide direct evidence that p21 controls autoreactive T cells, and to explore the therapeutic effect of p21 in lupus autoimmunity, we generated B6/*lpr* mice (homozygous for the Fas^{lpr} mutation, on a C57BL/6 background) that express the human p21 transgene in T cells, under the control of the proximal Lck promoter (B6/*lpr*-p21tg mice) (Daszkiewicz et al., 2015). p21 overexpression inhibited B6/*lpr* DN T cell lymphadenopathy and decreased effector/memory T cell proliferation and autoimmune symptoms. Further analysis showed that p21 overexpression reduced not only the proliferation, but also the activity of effector/memory B6/*lpr* T cells as well as their IFN-γ production. This result suggested that p21 has functions other than cell cycle inhibition in the control of T cell responses. Our first objective was therefore to study the role of p21 in effector/memory CD4$^+$ T cell activation and IFN-γ production.

T cell-directed p21 overexpression reduces effector/memory CD4$^+$ T cell activation and IFN-γ production in B6/*lpr* mice

To examine whether p21 overexpression affects the activation of effector/memory CD4$^+$ T cells in B6/*lpr*-p21tg mice, we analyzed surface expression of CD69, a T cell activation marker associated with memory T cells (Schoenberger, 2012). In accordance with the fact that the effector/memory T cell population in B6/*lpr* mice is hyperactivated, lymph node CD44^{hi}CD62L^{hi} CD4$^+$ B6/*lpr* T cells showed a marked increase (>4-fold) in CD69 expression compared to the equivalent B6 population (Figure 1A). In B6/*lpr*-p21tg mice, p21 overexpression reduced CD69 levels in the effector CD44^{hi}CD62L^{hi} population by ~50% (Figure 1A, upper panels), without affecting CD69 expression in memory CD44^{hi}CD62L^{lo} cells, which was similar in B6 and B6/*lpr* mice (Figure 1B, upper panels). As a control, we used B6-p21tg mice, which showed no exogenous p21 effect on CD69 expression in B6 effector/memory T cells (Figure 1A and B, lower panels). These data indicate that p21 overexpression reduces CD69 expression by hyperactivated effector B6/*lpr* T cells.

To test whether p21 overexpression had an effect on B6/*lpr* T cell function, we analyzed production of IFN-γ, a key cytokine associated with disease development in lupus models (Balomenos et al., 1998). The proportion of IFN-γ-producing cells was markedly increased in B6/*lpr* mice in both effector

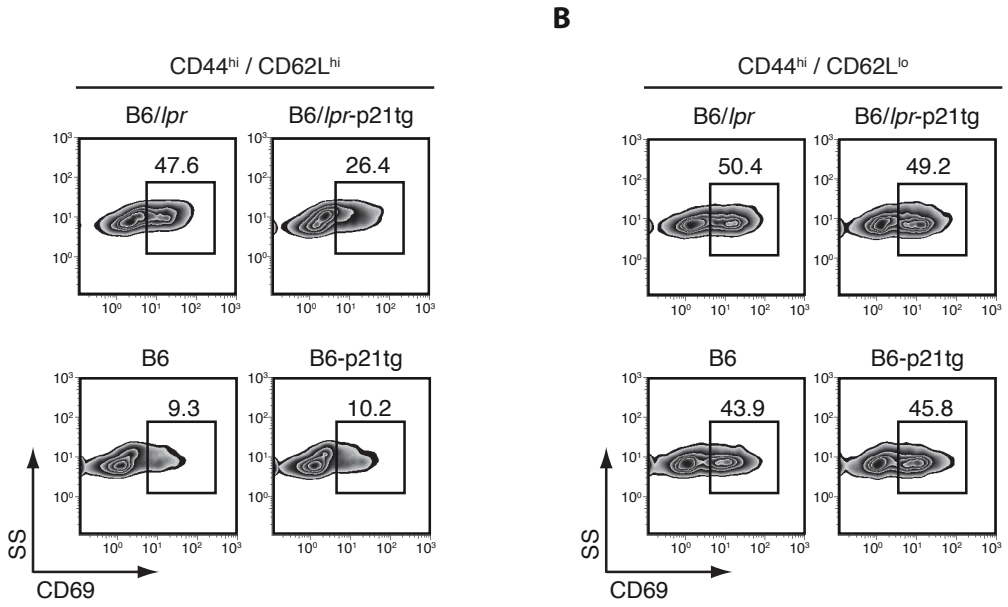


FIGURE 1 | p21 overexpression reduces activation of effector and memory CD4⁺ T cells in B6/*lpr*-p21tg mice. Surface expression of CD69 activation marker in lymph node CD62L^{hi}CD44^{hi} effector (**A**) and CD62L^{lo}CD44^{hi} memory (**B**) CD4⁺ T cells from 4-month-old B6/*lpr*, B6/*lpr*-p21tg, B6 and B6-p21tg mice, as determined by flow cytometry. Data show representative experiment of two performed (*n* = 4 mice).

CD44^{hi}CD62L^{hi} and memory CD44^{hi}CD62L^{lo} T cells compared with equivalent populations in B6 mice (Figure 2A and B). In B6/*lpr*-p21tg mice, p21 overexpression decreased the frequency of IFN-γ⁺ cells in effector and memory T cell populations (Figure 2A and B, upper panels), with no effect on B6-p21tg T cells (Figure 2A and B, lower panels). As memory T cell characterization is complex, we also analyzed another memory CD4⁺ T cell population defined as small CD44^{hi} cells, selected by forward scatter in flow cytometry (Rogers et al., 2000). Once again, we confirmed that p21 overexpression reduced memory T cell accumulation and IFN-γ production in B6/*lpr*-p21tg mice (Figure 2C, left panels), without affecting the equivalent B6-p21tg T cell population (Figure 2C, right panels). These data show that p21 overexpression reduces not only effector/memory T cell generation in B6/*lpr* mice, but also their activation and IFN-γ production.

p21 overexpression reduces IFN-γ production in B6/*lpr* T cells after repeated *in vitro* stimulation

After *in vitro* TCR stimulation and IL-2 expansion, secondary TCR stimulation leads to activation of the Fas/FasL system and apoptosis (AICD, activation-induced cell death). *lpr* T cells nonetheless have defective AICD and hyperproliferate when restimulated *in vitro* (Singer et al., 1994). In addition to reducing B6/*lpr*-p21tg T cell hyperproliferation after secondary TCR stimulation, p21 overexpression also reduced conversion of memory to effector cells, suggesting that the p21 transgene negatively regulates B6/*lpr* T cell activation (Daszkiewicz et al., 2015). To corroborate these data, we analyzed whether p21 overexpression affects IFN-γ production. After initial TCR stimulation and IL-2 expansion, we stimulated the cells with IL-12 and IL-18, which are known to synergistically induce IFN-γ production (Robinson et al., 1997). As a result, B6/*lpr* T cells showed a high capacity to produce IFN-γ, whereas in B6/*lpr*-p21tg T cells, p21 transgenic expression significantly

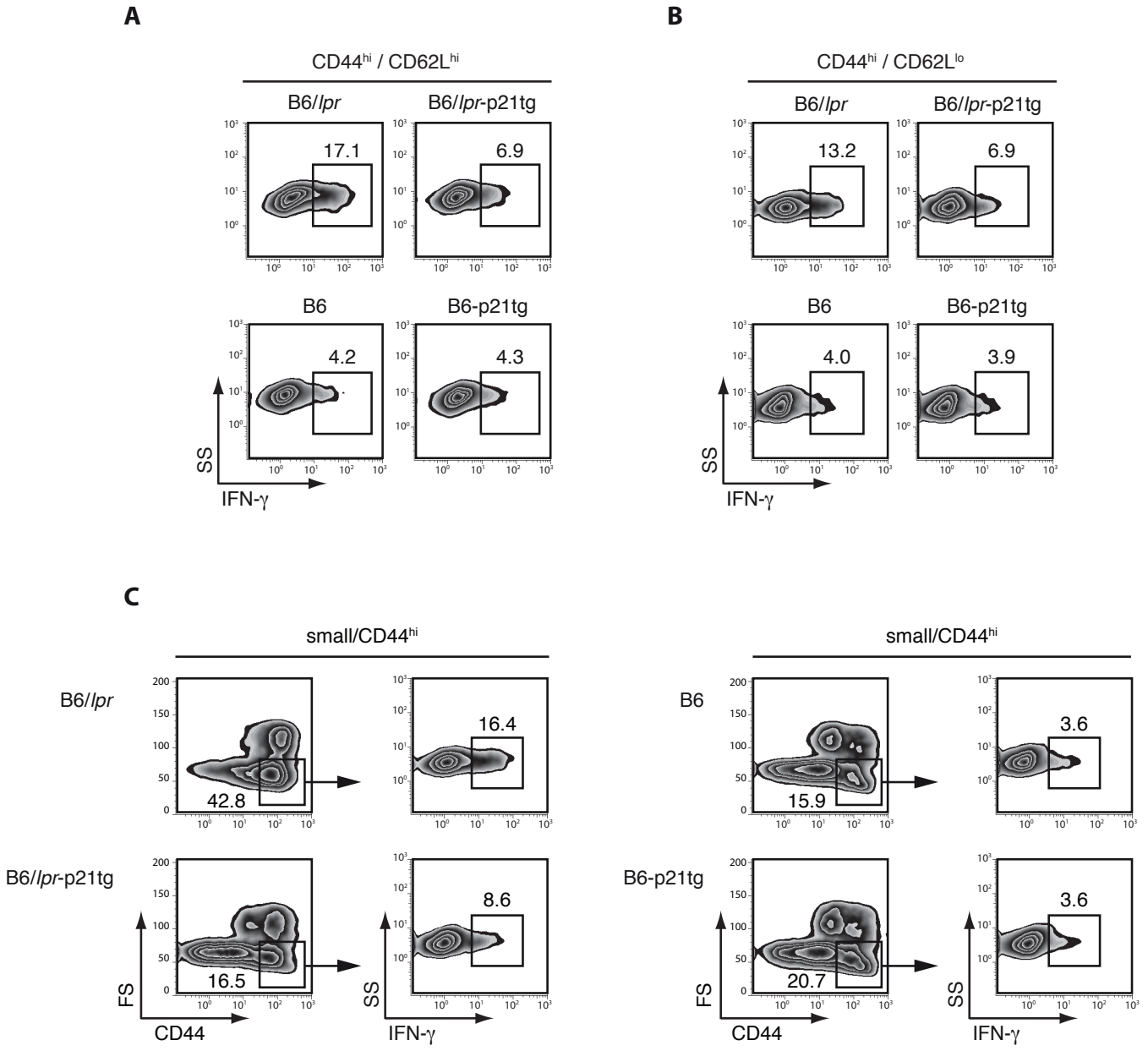


FIGURE 2 | **p21 overexpression reduces IFN-γ production in B6*lpr*-p21tg effector and memory T cells.** Lymph node cells from 4-month-old B6/*lpr*, B6/*lpr*-p21tg, B6 and B6-p21tg mice were stimulated *ex vivo* with PMA (50 ng/ml) and ionomycin (2 μ/ml) for 4h and analyzed for intracellular IFN-γ. Flow cytometry analysis showing the frequency of IFN-γ-producing CD62L^{hi}CD44^{hi} effector (**A**) and CD62L^{lo}CD44^{hi} memory (**B**) CD4^{+} T cells. (**C**) The frequency of IFN-γ-producing small CD44^{hi} CD4^{+} memory T cells in B6/*lpr*, B6/*lpr*-p21tg, B6 and B6-p21tg mice. Data show representative experiment of two performed (*n* = 4 mice).

reduced the proportion of IFN-γ-producing cells (Figure 3). These data show that p21 overexpression suppresses IL-12- and IL-18-dependent IFN-γ production in effector/memory CD4^{+} T cells, and suggest that p21 could control intrinsic T cell activation independently of TCR stimulation.

Reduced IFN-γ production in B6/*lpr*-p21tg T cells has a paracrine effect on macrophage activation

T cell-directed p21 overexpression in B6/*lpr* mice reduced the proportions of IFN-γ-secreting effector/memory T cells, thereby reducing autoantibody production and disease

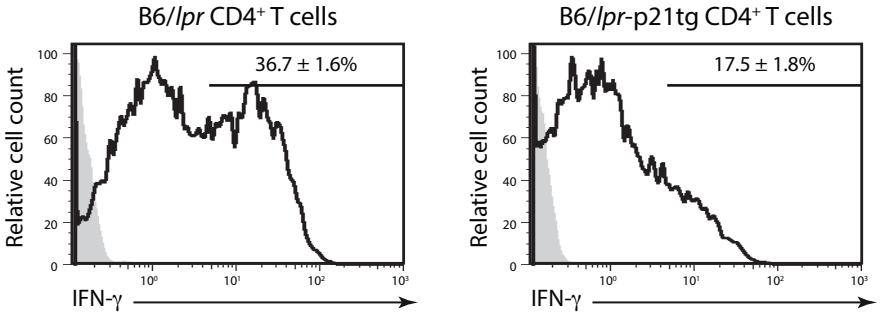


FIGURE 3 | **p21 overexpression decreases IFN-γ induction by IL-12 and IL-18 in B6*lpr*-p21tg effector/memory T cells after *in vitro* repeated stimulation.** CD4+ T cells from B6/*lpr* and B6/*lpr*-p21tg mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 in the presence of IL-2 for 24 h. Flow cytometry analysis showing the frequency of IFN-γ-producing effector/memory CD4+ T cells. Data show mean ± SEM (n = 3); p = 0.0013, two-tailed Student's t test.

manifestations in these mice (Daszkiewicz et al., 2015). As macrophage activation is critical for *lpr* disease development (Carvalho-Pinto et al., 2002), we investigated whether p21 overexpression in T cells has a paracrine effect on macrophage activation. Peritoneal B6/*lpr* macrophages were exposed to culture supernatants of repeatedly stimulated B6/*lpr* and B6/*lpr*-p21tg T cells. The IFN-γ concentration in T cell culture supernatants was strongly reduced in the presence of the p21 transgene (Figure 4A). Whereas B6/*lpr* T cell supernatants strongly activated macrophages, seen as high STAT1 phosphorylation and iNOS protein levels, equivalent supernatants from B6/*lpr*-p21tg T cells induced very low p-STAT1 levels and iNOS was undetectable (Figure 4B). These data suggest that large amounts of IFN-γ are needed to induce iNOS, which was confirmed by stimulating macrophages at different IFN-γ concentrations (Figure 4B). The results highlight the potential of p21 overexpression to control disease in a paracrine fashion.

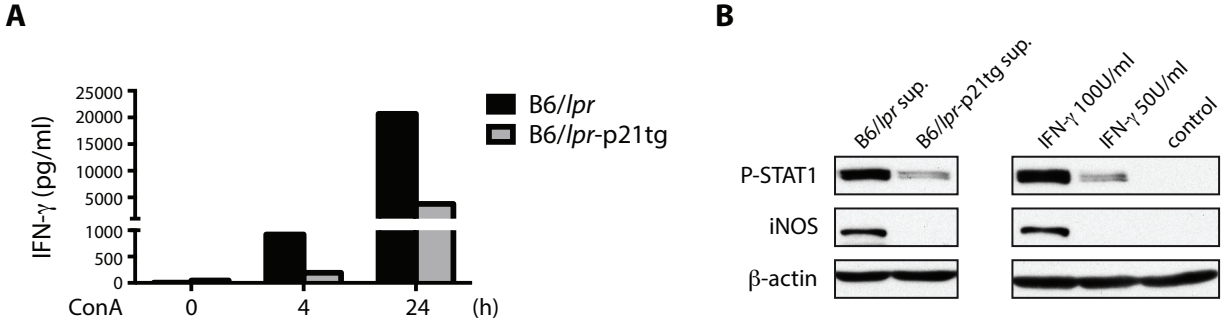


FIGURE 4 | **Decreased B6/*lpr* macrophage activation by B6/*lpr*-p21tg T cell culture supernatant.** (**A**) IFN-γ concentrations in supernatants from B6/*lpr* and B6/*lpr*-p21tg T cell cultures after secondary ConA stimulation. (**B**) Left, Immunoblot analysis showing STAT1 phosphorylation and iNOS protein levels in B6/*lpr* peritoneal macrophages exposed to supernatants from B6/*lpr* and B6/*lpr*-p21tg T cells repeatedly stimulated with ConA. Right, p-STAT1 and iNOS levels in B6/*lpr* peritoneal macrophages after stimulation with different concentrations of IFN-γ. Shown is representative experiment of 3 performed.

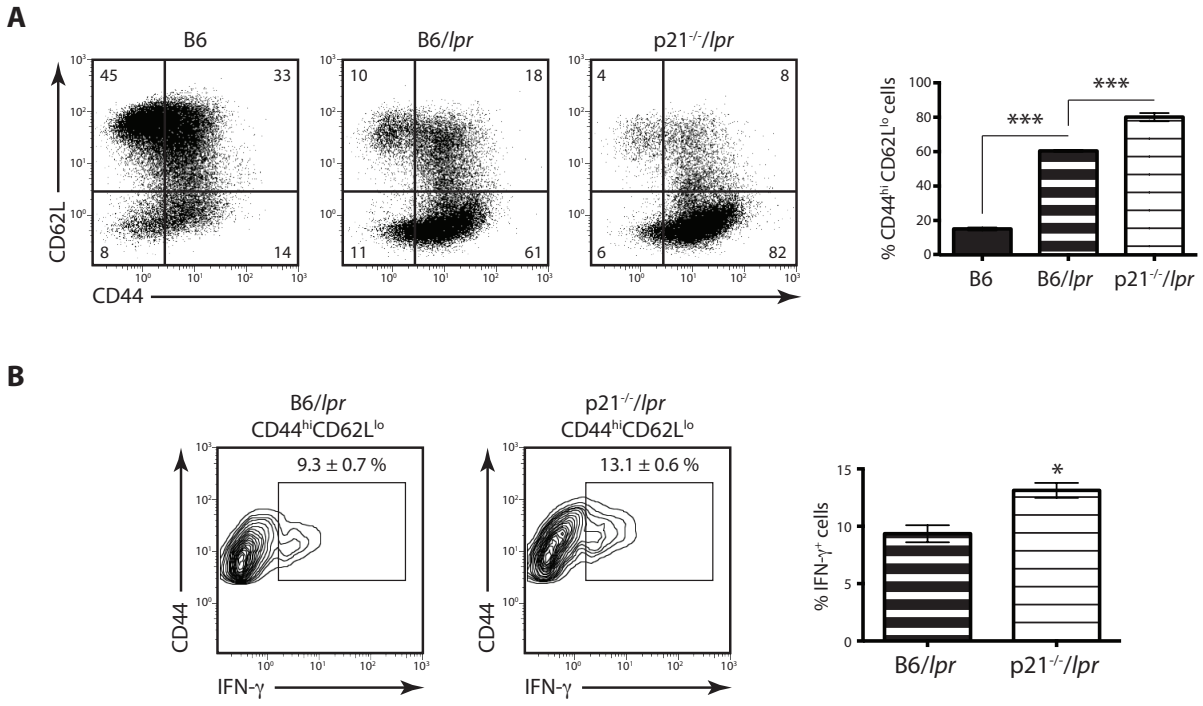


FIGURE 5 | Lack of p21 further enhances the accumulation of IFN-γ-producing B6/*lpr* memory T cells. CD4$^+$ T cells from B6, B6/*lpr* and p21$^{-/-}$/*lpr* mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 in the presence of IL-2 for 24 h. (**A**) Flow cytometry analysis showing surface expression of CD62L and CD44 in CD4$^+$ T cells. Data show mean ± SEM (n = 3); ***p<0.001, one-way ANOVA with Bonferroni correction. (**B**) The frequency of IFN-γ-producing CD44^{hi}CD62L^{lo} memory CD4$^+$ T cells. Data show mean ± SEM (n = 3); *p<0.05, two-tailed Student's t test.

Lack of p21 increases B6/*lpr* effector/memory CD4$^+$T cell generation and IFN-γ production

Since our results indicated that p21 expression is important in the control of apoptosis-resistant memory T cell proliferation and activation, we generated p21-deficient B6/*lpr* mice to determine whether the lack of p21 has an effect on the hyperactivation status of B6/*lpr* T cells. Indeed, p21$^{-/-}$B6/*lpr* mice showed even higher effector/memory CD4$^+$ T cell activation and accumulation than B6/*lpr* mice; this aggravated autoimmune pathology in the p21$^{-/-}$B6/*lpr* mice, which was only mild in the B6/*lpr* mice, and led to increased mortality (our unpublished observations). We therefore examined the activation status of these cells and their IFN-γ-producing potential after *in vitro* repeated stimulation. After a second stimulation with IL-12 and IL-18, B6/*lpr* T cells showed increased proportions of CD44^{hi}CD62L^{lo} memory cells compared with B6 mice, which showed a predominant naïve CD44^{lo}CD62L^{hi} phenotype (Figure 5A). Strikingly, the p21-deficient mice showed further increased proportions of CD44^{hi}CD62L^{lo} memory T cells in comparison with B6/*lpr* mice (Figure 5A). We next analyzed the activation of these cells and found significantly increased IFN-γ production in CD44^{hi}CD62L^{lo} p21$^{-/-}$B6/*lpr* compared with B6/*lpr* memory T cells (Figure 5B). p21 deficiency thus increases generation of Fas-deficient effector/memory T cells as well as their IFN-γ-producing potential.

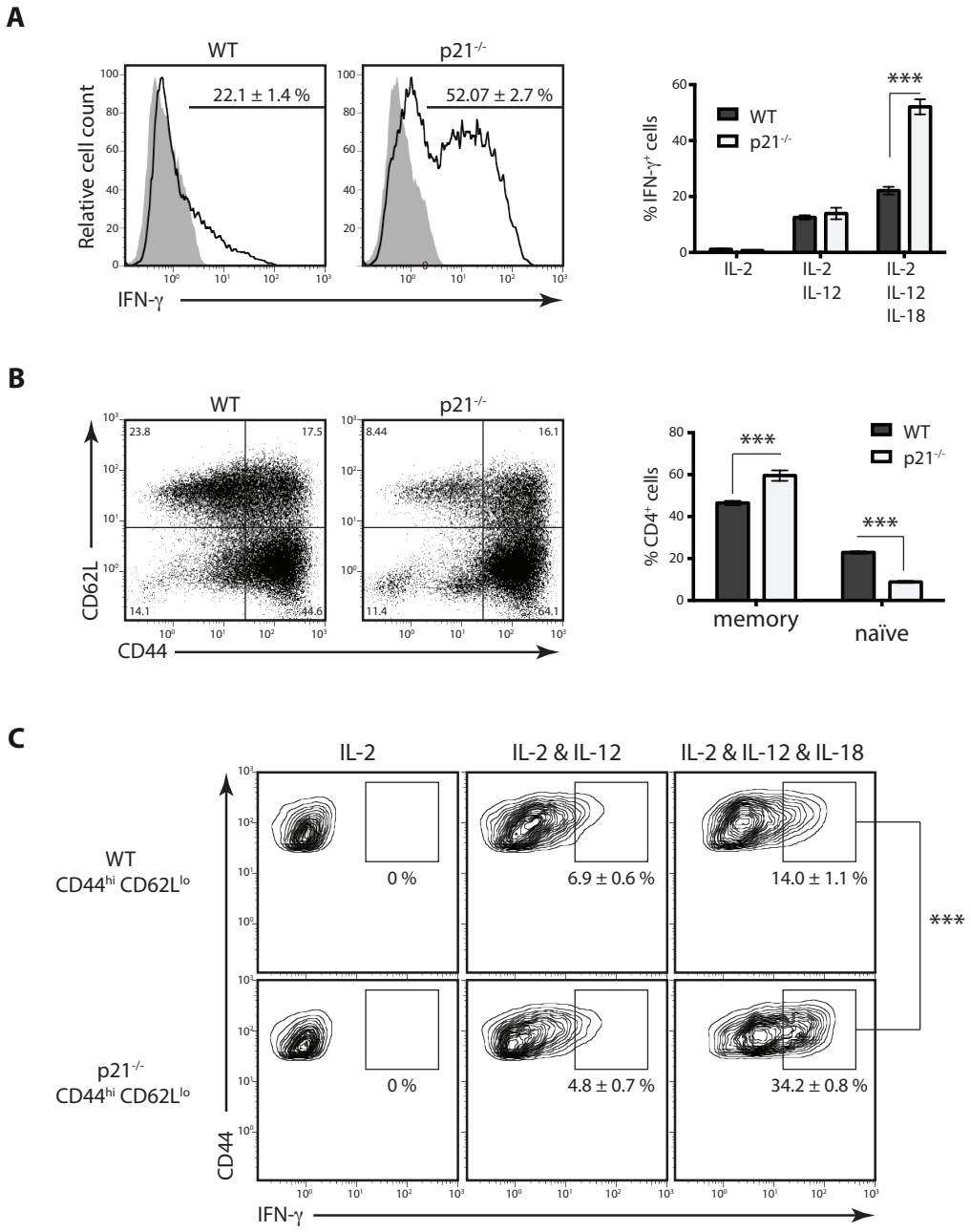


FIGURE 6 | Lack of p21 increases IFN-γ production in memory CD4⁺ T cells after *in vitro* repeated stimulation. CD4⁺ T cells from WT and p21$^{-/-}$ mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 in the presence of IL-2 for 24 h. Control cells were restimulated with IL-12 and IL-2 or only IL-2. (**A**) Frequency of IFN-γ-producing WT and p21$^{-/-}$ CD4⁺ T cells. Cells were stained for CD4 expression and intracellular IFN-γ and analyzed by flow cytometry. (**B**) Flow cytometry analysis showing the frequency of CD62L^{hi}CD44^{lo} (naïve) and CD62L^{lo}CD44^{hi} (memory) WT and p21$^{-/-}$ CD4⁺ T cells at 24 h after restimulation with IL-12 and IL-18. (**C**) Frequency of IFN-γ-producing CD62L^{lo}CD44^{hi} memory WT and p21$^{-/-}$ CD4⁺ T cells. Data show mean ± SEM (n = 3); **p<0.01; ***p<0.001, two-way ANOVA with Bonferroni correction.

Elevated IFN-γ production by p21$^{-/-}$ effector/memory CD4⁺ T cells

To study whether p21 controls IFN-γ production in effector/memory CD4⁺ T cells independently of lack of Fas, we examined IL-12- and IL-18-dependent IFN-γ induction in WT (B6) and p21$^{-/-}$ effector/memory T cells obtained after *in vitro* TCR-dependent stimulation and IL-2 expansion. While IL-12 combined with IL-18 induced significantly higher levels of IFN-γ in p21$^{-/-}$ compared with WT effector/memory T cells (Figure 6A, left

panels), the levels of IFN-γ induced by IL-12 alone were similar in WT and p21$^{-/-}$ cells (Figure 6A, right panel). These data suggested that p21 negatively controls the signaling downstream of IL-18 and not of IL-12. Further analysis of the effector/memory T cell subsets revealed that lack of p21 led to increased generation of CD44^{hi}CD62L^{lo} memory cells, which resulted in significantly lower proportions of naïve CD44^{lo}CD62L^{hi} cells in comparison with WT cells (Figure 6B). To exclude the possibility that increased IFN-γ production is a result of the increased percentage of p21$^{-/-}$ CD44^{hi}CD62L^{lo} cells, we analyzed IFN-γ production within the CD44^{hi}CD62L^{lo} gate. After IL-12/IL-18 stimulation, lack of p21 significantly increased the proportions of IFN-γ-producing CD44^{hi}CD62L^{lo} memory T cells compared with WT cells, with no such effect during IL-12 stimulation alone (Figure 6C). Deficiency in p21 therefore increases the generation of effector/memory T cells and their activation via IL-18.

Lack of p21 increases NF-κB DNA binding activity in effector/memory CD4^{+} T cells

To obtain mechanistic insight into how p21 regulates IFN-γ production, we analyzed the IL12/IL-18-dependent activation of STAT4 and NF-κB, respectively (Robinson et al., 1997; Nakahira et al., 2002). Electrophoretic mobility shift assay (EMSA) analysis showed markedly increased NF-κB activation in p21$^{-/-}$ compared with WT effector/memory T cells at 1 h post-IL-12/IL-18 stimulation (Figure 7A). We also detected high levels of phosphorylated STAT4 in p21$^{-/-}$ effector/memory T cells at 2 h post-IL-12/IL-18 stimulation (Figure 7B). This was unexpected, since p21 expression had no effect on IFN-γ production induced by IL-12 alone. High NF-κB activation and/or IFN-γ production induced by IL-18 contributes to increased IL-12 receptor expression and consequently, STAT4 phosphorylation (Aita et al., 2004; Wu et al., 2000). Lack of p21 therefore enhances IFN-γ production in effector/memory CD4^{+} T cells through its effect on IL-18-dependent NF-κB activation.

p21 regulates the production of mROS in effector/memory CD4^{+} T cells

Signaling events dependent on mitochondrial reactive oxygen species (mROS) are needed for correct TCR-dependent activation and IL-2 production (Sena et al., 2013). We therefore

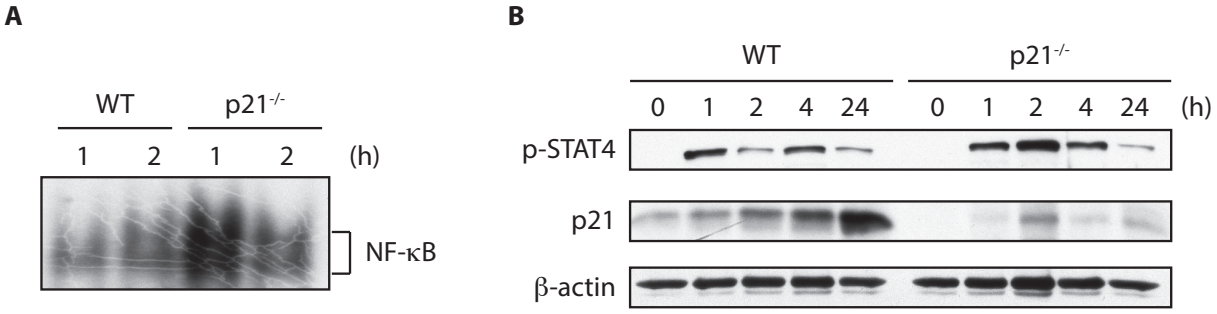


FIGURE 7 | **Increased STAT4 and NF-κB activation in effector/memory T cells in absence of p21.** CD4^{+} T cells from WT and p21$^{-/-}$ mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 in the presence of IL-2 for 24 h. (**A**) EMSA analysis of nuclear extracts showing NF-κB protein complexes binding the consensus DNA sequence. (**B**) Immunoblot showing phospho-STAT4 and p21 protein levels in whole cell lysates. Equal loading is visualized by β-actin expression. Data show representative experiment of two performed.

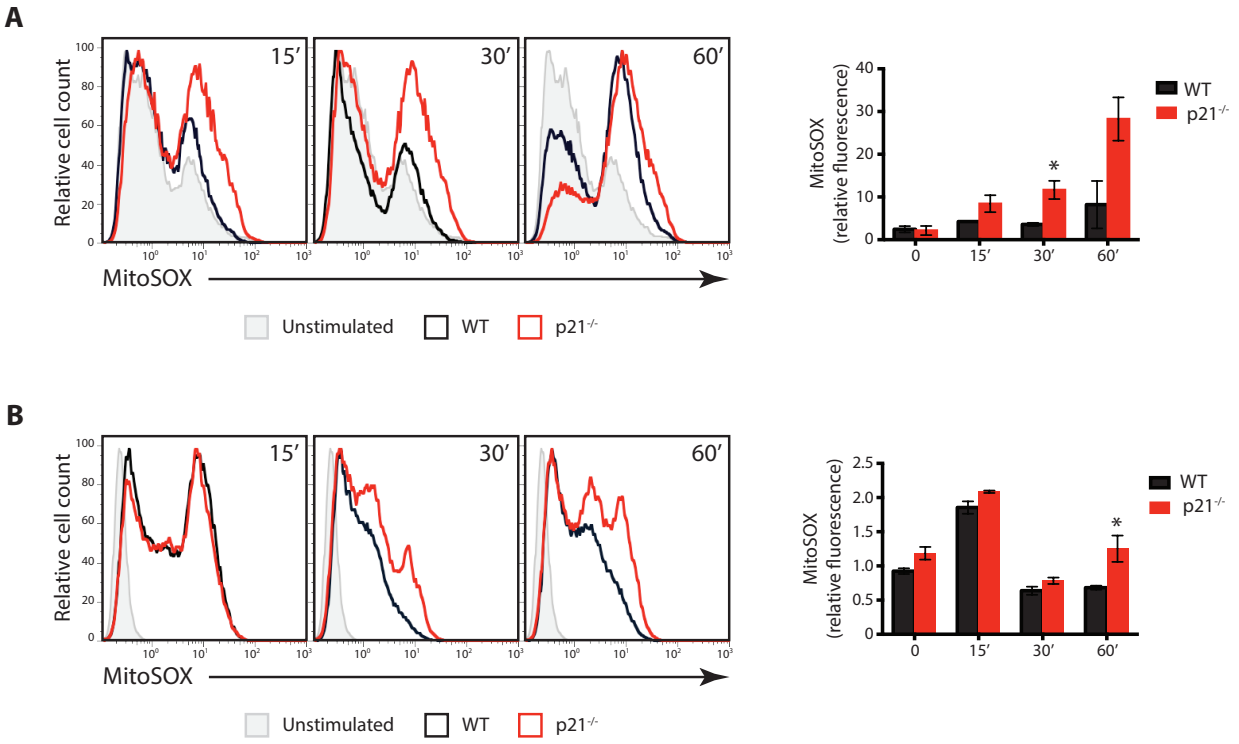


FIGURE 8 | **Mitochondrial superoxide production is increased in effector/memory T cells in absence of p21.** Flow cytometry analysis of mROS production measured by mitoSOX Red fluorescence. $CD4^+$ T cells from WT and $p21^{-/-}$ mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 (**A**) or ConA (**B**) for indicated time points. Histograms show representative staining. Median fluorescence intensities were normalized to the unstimulated cells of each genotype. Graphs show mean ± SEM (n = 3); *p<0.05, two-way ANOVA with Bonferroni correction.

considered that p21 might regulate NF-κB activation and IFN-γ production in effector/memory T cells through an effect on mROS.

We first examined IL-12/IL-18-dependent mROS induction in WT and $p21^{-/-}$ effector/memory T cells obtained after *in vitro* TCR-dependent stimulation and IL-2 expansion. Using the MitoSOX Red dye, which detects mitochondrial superoxide production in live cells, we found that $p21^{-/-}$ effector/memory T cells showed elevated mROS production compared with WT cells, at early times after IL-12/IL-18 stimulation (Figure 8A). To test whether p21 expression affects mROS production after TCR-dependent stimulation, we performed a second stimulation with concavalin A (ConA). mROS production was again clearly elevated in $p21^{-/-}$ effector/memory compared with WT T cells, at 30 and 60 min post-ConA restimulation (Figure 8B). Finally, we performed antigen-specific stimulation using transgenic T cells expressing a TCR specific for chicken ovalbumin (OVA), which were isolated from OT-II and $p21^{-/-}$OT-II mice (Robertson et al., 2000). These T cells were stimulated *in vitro* in the presence of OVA-presenting dendritic cells, expanded for 6 days with IL-2, and restimulated with the same cells (Figure 9A). Early after second antigen-specific stimulation, p21 deficiency led to elevated mROS production in effector/memory T cells (Figure 9B). Collectively, these data show that lack of p21 leads to increased mROS production in effector/memory T cells, after both TCR-dependent and -independent stimulation.

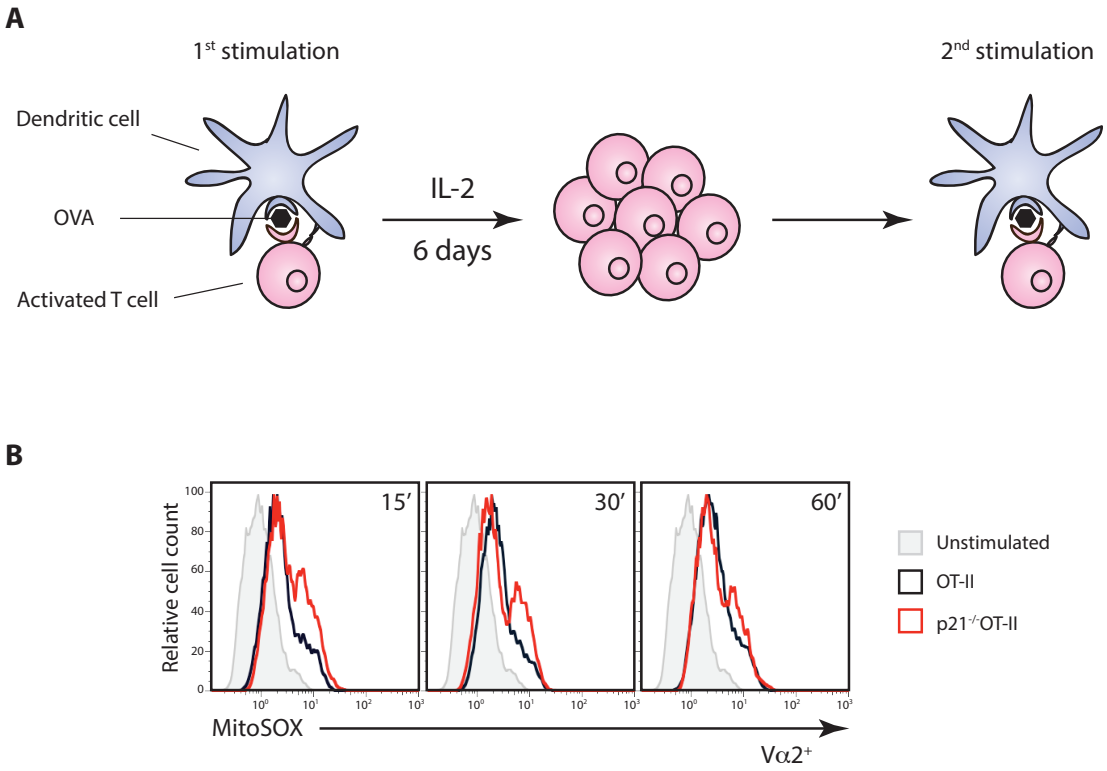


FIGURE 9 | Increased mitochondrial superoxide production after antigen-specific stimulation of p21-deficient effector/memory T cells. (**A**) CD4+ T cells from OT-II and p21-/-OT-II mouse spleens were incubated with OVA-presenting dendritic cells for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with OVA-presenting dendritic cells. (**B**) Flow cytometry analysis of mROS production measured by mitoSOX Red fluorescence at indicated time points of secondary stimulation. Histograms show representative staining. Median fluorescence intensities were normalized to the unstimulated cells of each genotype. Shown is representative experiment of three performed.

Inhibition of mitochondrial ROS reduces IFN-γ production in CD4+ effector/memory T cells

To determine whether mROS are functionally important for IL-12/IL-18-induced IFN-γ production, we treated the cells with mROS inhibitor prior to the second stimulation. The major source of ROS is mitochondrial oxidative phosphorylation, as electron leakage occurs at complex I, complex II and complex III, converting oxygen to superoxide (Li and Trush 1998). DPI (diphenyleneiodonium) blocks mROS by inhibiting FAD (flavin adenine dinucleotide), which is present at both mitochondrial complex I (NADH-quinone oxidoreductase) and complex II (succinyl dehydrogenase) (Figure 10A). Using the MitoSOX Red dye, we found that DPI treatment significantly reduced mROS levels in both WT and p21-/- effector/memory T cells after second stimulation with IL-12/IL-18 (Figure 10B). mROS levels were similar in WT and p21-/- cells after DPI treatment, indicating that lack of p21 might increase mROS through complex I or complex II (Figure 10B). IFN-γ production was severely reduced by DPI treatment in both WT and p21-/- effector/memory T cells (Figure 10C). These data identify mROS as an important signaling component for IL-12/IL-18-induced IFN-γ production, both in normal and in p21-deficient hyperactivated effector/memory CD4+ T cells, and point to p21 as a regulator of mitochondrial complexes I and II.

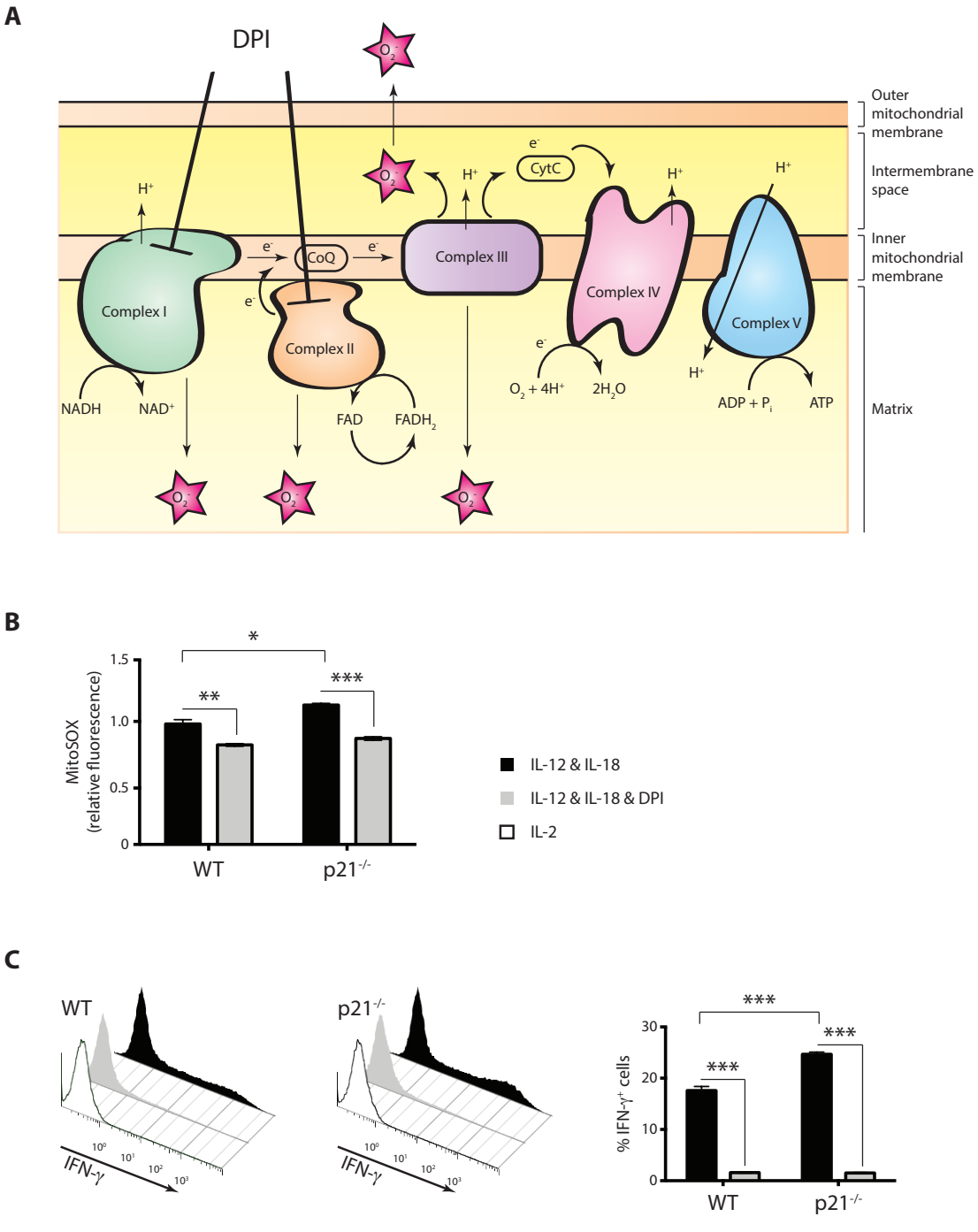


FIGURE 10 | Inhibition of mitochondrial ROS reduces normal IFN-γ production and reverts high IFN-γ production in p21-deficient effector/memory CD4⁺ T cells. (**A**) Prior to secondary stimulation with IL-12 and IL-18, WT and p21$^{-/-}$ CD4⁺ T cells were incubated with mitochondrial complex I and complex II inhibitor DPI. (**B**) DPI treatment reduced mROS at 60 min post-secondary stimulation. (**C**) Reduced frequency of IFN-γ-producing CD4⁺ T cells after DPI treatment. Histograms show representative staining. Graphs show mean ± SEM (n = 3); *p<0.05; **p<0.01; ***p<0.001, two-way ANOVA with Bonferroni correction.

Mitochondrial ROS promote the accumulation of CD44^{hi} effector/memory T cells

Mitochondrial metabolism has an important role in memory T cell differentiation (Pearce et al., 2009). We therefore evaluated whether p21 regulates effector/memory T cell generation, through its effect on mROS production. To assess the capacity of WT and p21$^{-/-}$ CD4⁺

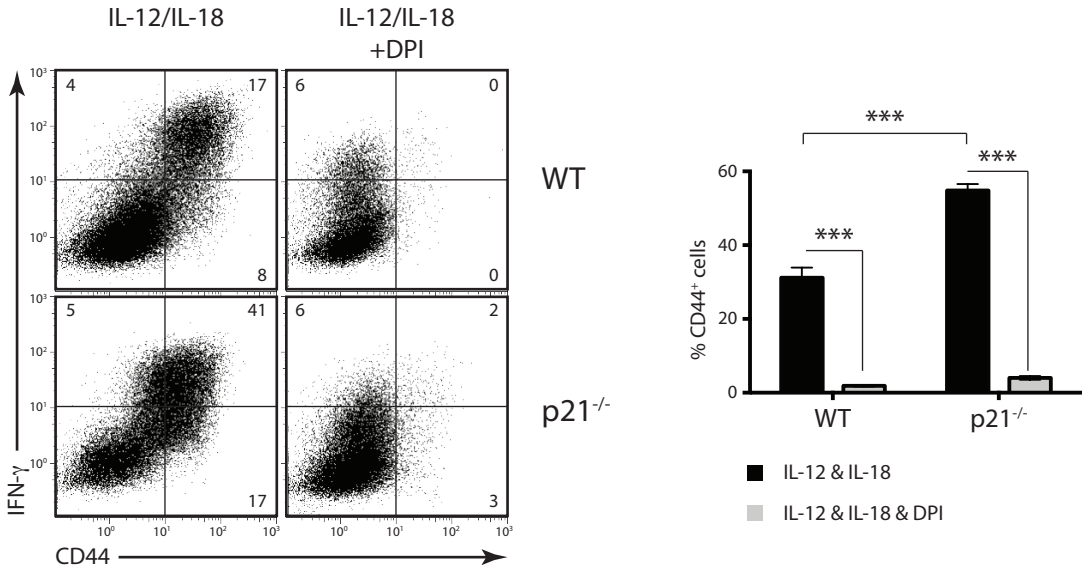


FIGURE 11 | Inhibition of mitochondrial ROS reduces CD44⁺ effector/memory T cell accumulation. CD4⁺ T cells from WT and p21$^{-/-}$ mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 alone or in the presence of indicated antioxidants for 24 h. CD44 expression was determined by flow cytometry. Representative dot plots are shown. Graphs show mean ± SEM (n = 3); ***p<0.001, two-way ANOVA with Bonferroni correction.

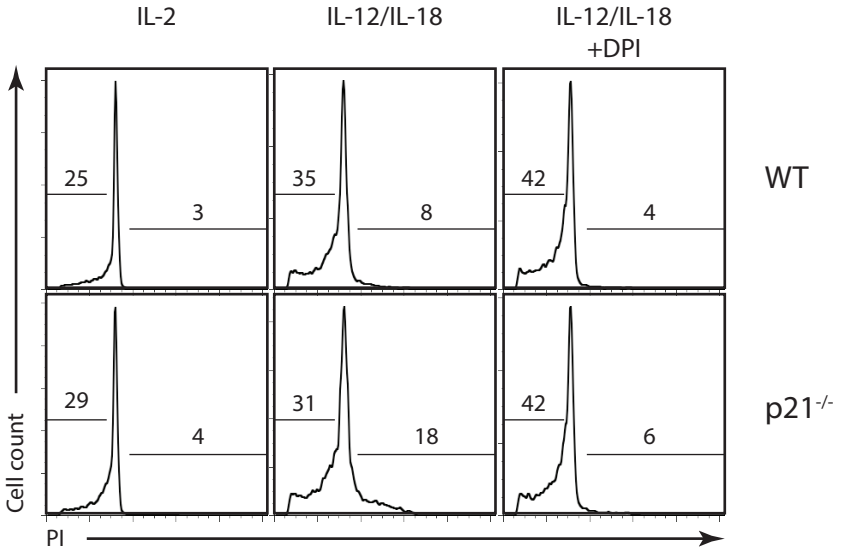


FIGURE 12 | DPI treatment does not affect cell viability. Cell cycle analysis of B6 and p21$^{-/-}$ effector/memory CD4⁺ T cells at 24 h post-secondary stimulation with IL-12 and IL-18. Representative data are shown (n = 3).

T cells to acquire memory phenotype, we analyzed CD44 expression after second stimulation with IL-12/IL-18. As shown above (Figures 5 and 6), lack of p21 led to increased accumulation of CD44^{hi} effector/memory T cells (~30 *vs.* ~60%), and increased the percentage of IFN-γ-producing CD44^{hi} memory cells (~20 *vs.* ~40%) compared with WT cells (Figure 11). In addition to reducing the capacity of these cells to produce IFN-γ (Figure 10C and Figure 11), DPI treatment severely altered their CD44^{hi} memory phenotype (Figure 11). We observed no marked effect of DPI treatment on cell viability (Figure 12). These results identify mROS as a driver of memory T cell differentiation, and suggest that p21 controls memory T cell accumulation and activation by regulating mROS.

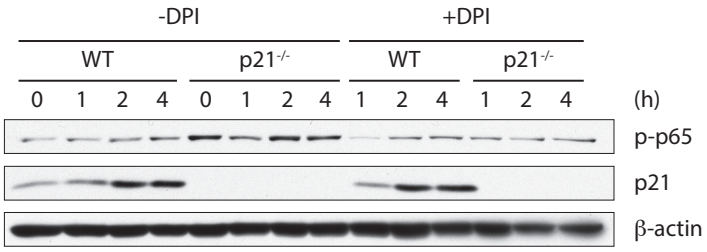


FIGURE 13 | Inhibition of mitochondrial ROS reverts high NF-κB activation in p21-deficient effector/memory CD4⁺ T cells. CD4⁺ T cells from WT and p21$^{-/-}$ mouse spleens were stimulated with ConA for 24 h, expanded in presence of IL-2 for 6 days, and restimulated with IL-12 and IL-18 alone or in the presence of DPI for indicated times. Immunoblot analysis shows phosphorilated p65 protein levels in whole cell extracts. p21 expression was not affected by DPI treatment. Equal loading is visualized by β-actin expression. Data show representative experiment of two performed.

p21 deficiency increases TCR-dependent NF-κB stimulation through mitochondrial ROS

p21 regulates effector/memory T cell activation after repeated *in vitro* TCR stimulation (Daszkiewicz et al., 2015). Our results identified p21 as a regulator of mROS production after repeated TCR stimulation (Figures 8 and 9). We therefore tested whether these increased mROS in p21$^{-/-}$ cells have an effect on NF-κB activation downstream of the TCR. After a second TCR stimulation, we detected elevated p65 phosphorylation in p21$^{-/-}$ compared with WT effector/memory T cells (Figure 13). After DPI treatment, p65 phosphorylation was reduced in p21$^{-/-}$ effector/memory T cells, and was reverted to the levels of WT cells (Figure 13). p21 protein levels were unaffected by DPI treatment (Figure 13), which showed that p21 acts upstream of mROS. These findings identify p21 as an important regulator of mROS production during effector/memory T cell activation. This role of p21 is independent of its CDK2 inhibitor function (our unpublished data).

p21 deficiency increases antigen-specific CD4⁺ T cell-dependent inflammation *in vivo*

To determine whether p21 deficiency would affect the progression of a CD4⁺ T cell-dependent inflammatory disease, we used the OVA model of allergic asthma. Lung inflammation in this model is entirely dependent on CD4⁺ T cells (Afshar et al., 2008). WT and p21$^{-/-}$ mice were sensitized with OVA plus alum at day 0 and day 10, and then aerosol challenged daily from day 19 to day 24 for 20 min with OVA, and sacrificed 24 h after the final treatment for analysis of lung inflammation (Bryce et al., 2006). Control mice were sensitized with alum alone and aerosol challenged with PBS. In WT mice, histological analysis of the lung revealed mild peribronchiolar and perivascular infiltration of macrophages, lymphocytes, eosinophils and neutrophils. In contrast, p21-deficient mice had severe pneumonitis with striking perivascular and interstitial inflammation, which suggested that p21 is necessary to control the level of chronic inflammation in lungs (Figure 14A). In accordance with this, circulating levels of OVA-specific immunoglobulin E (IgE) were significantly increased in p21$^{-/-}$ compared with WT mice (Figure 14B). IgE production is a B cell function that requires assistance from CD4⁺ T cells for activation and class switching. Elevated IgE production in p21$^{-/-}$ mice therefore concurs with their CD4⁺ T cell hyperactivation, although we cannot rule out the possibility of intrinsic hyperactivation of p21$^{-/-}$ B cells. In any case, this model provides evidence that p21 is an essential regulator of the immune response, not only in the context of autoimmunity, but also in other settings such as lung inflammation.

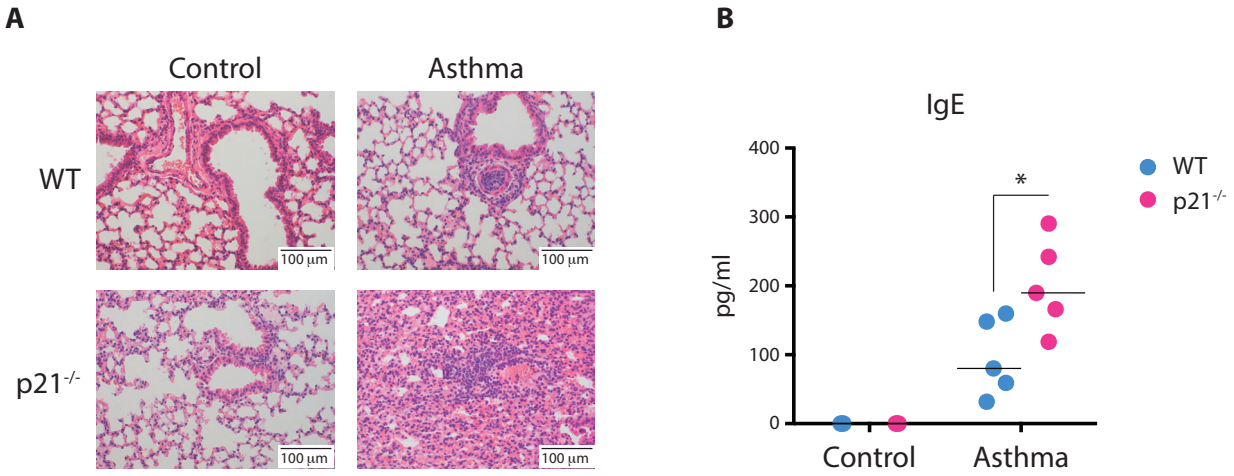


FIGURE 14 | **p21 deficiency aggravates CD4⁺ T cell-dependent airway inflammation *in vivo*.** (**A**) Lung sections subjected to hematoxylin and eosin staining showing impressive peribronchiolar and perivascular inflammation in p21$^{-/-}$ mice compared with WT after OVA aerosol challenge. (**B**) Serum OVA-specific IgE levels as measured by sandwich ELISA. Data show median (n = 5 mice); *p<0.05, Mann-Whitney test, U = 2.

Chapter 2: p21 regulates LPS-induced mitochondrial reactive oxygen species and DNA binding of p65/p50 NF-κB

A previous study from our laboratory demonstrated that p21 negatively regulates IKK complex activity in LPS-activated macrophages (Trakala et al., 2009), and therefore, NFκB; the underlying mechanism nonetheless remained poorly understood. TLR4 activation leads to TRAF6-dependent activation of the TAK1 kinase, which in turn activates two distinct pathways involving the IKK complex and MAPK (ERK, JNK and p38) (Kawai and Akira, 2007). A recent study showed that TRAF6 can translocate to the mitochondria, where it interacts with proteins involved in the mitochondrial respiratory chain, leading to increased mitochondrial and cellular ROS generation (West et al., 2011a). Mounting evidence indicates that mROS, induced by TLR4 activation, facilitate bacterial phagocytosis and pro-inflammatory cytokine production through redox-sensitive activation of the IKK complex and NF-κB (Bai et al., 2005; Gloire et al., 2006; West et al., 2011b). We therefore analyzed whether p21 deficiency affects mROS generation in LPS-activated macrophages, leading to elevated NF-κB activity.

p21 deficiency increases generation of mitochondrial and cellular reactive oxygen species in LPS-activated macrophages

Consistent with previous reports, LPS stimulation vigorously upregulated p21 expression in WT peritoneal macrophages, at early times post-stimulation (Figure 15A, left). p21 levels reached a peak at 2 h post-LPS challenge and remained high at 4 h, as confirmed by RT-PCR analysis (Figure 15A, right). We next examined the levels of cellular ROS in LPS-activated bone marrow-derived macrophages (BMDM), using the CM-H_2DCFDA indicator, which detects total cellular H_2O_2 in live cells. Cellular ROS levels were notably increased as early as 15 min after LPS activation, and remained significantly higher in p21-deficient macrophages than in WT controls until 1 h post-LPS challenge (Figure 15B). Using the MitoSOX Red dye, which detects mitochondrial superoxide production in live cells, we found elevated mROS production, which peaked as early as 15 min post-LPS stimulation, and remained higher in $p21^{-/-}$ macrophages than in WT controls (Figure 15C). Overall, these data show that after LPS stimulation, lack of p21 leads to increased mitochondrial superoxide production and accumulation of cellular H_2O_2, which could account for the increased IKK complex activity, leading to more vigorous IκBα degradation and higher NF-κB binding activity in $p21^{-/-}$ compared with WT macrophages.

p21 deficiency affects IκBα degradation, but not MAPK or IRF3 activation in LPS-stimulated macrophages

To understand how lack of p21 mediates increased NF-κB activation, and whether increased mitochondrial ROS activate other signaling molecules in the absence of p21, we examined the activation of MyD88-dependent and independent pathways, which are activated by TLR4 stimulation. There was no detectable difference in interferon regulatory factor 3 (IRF3)

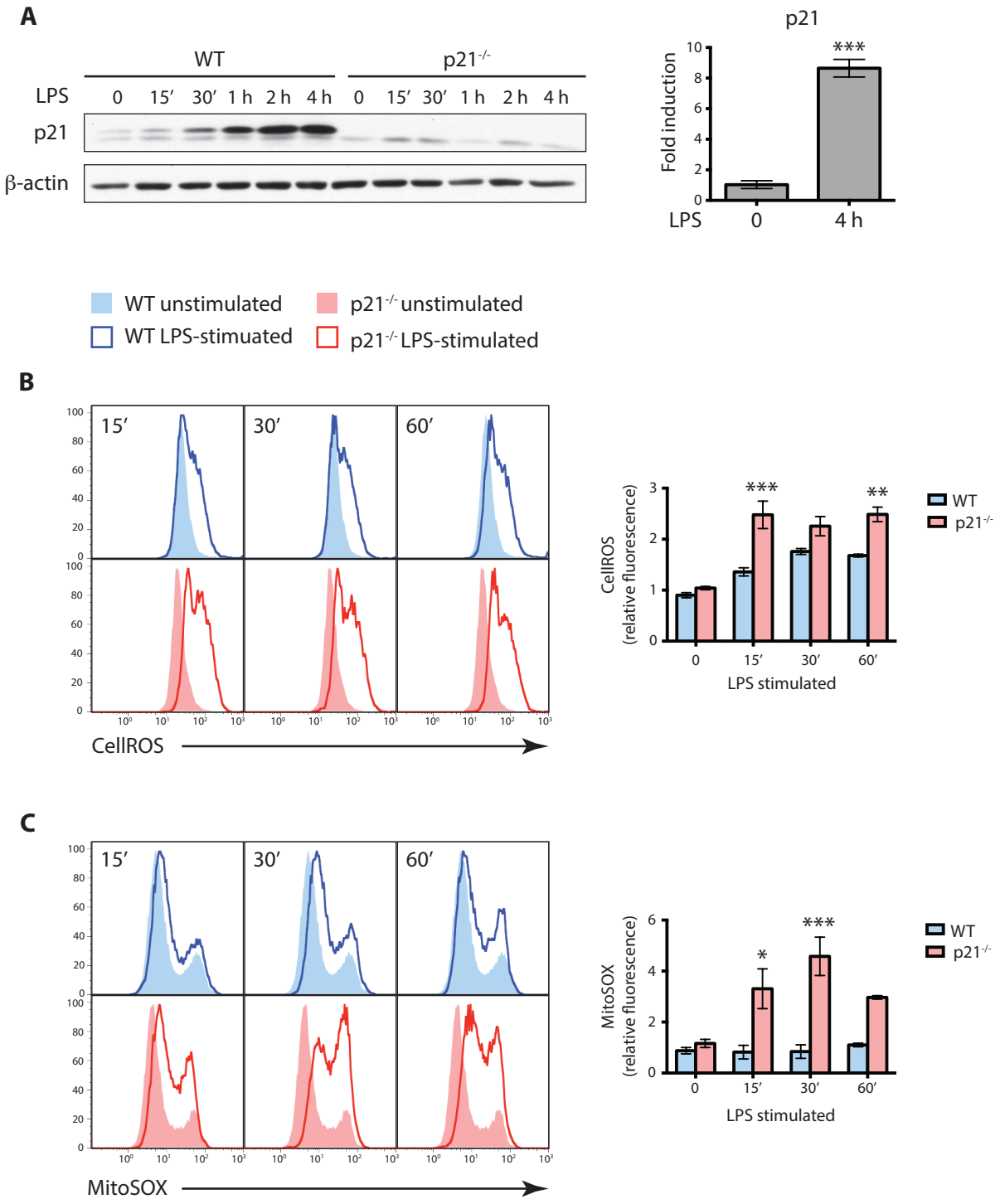


FIGURE 15 | Cellular and mitochondrial ROS are augmented in LPS-activated macrophages in absence of p21. (**A**) WT and p21$^{-/-}$ BMDMs were stimulated with 100 ng/ml LPS for indicated times and analyzed by Western blot (left) and RT-PCR (right) for p21 expression. β-actin is used as a housekeeping control. Data show mean ± SEM (n = 3); ***p<0.001, two-tailed Student's t test. Flow cytometry analysis of cellular H_2O_2 levels measured by CM-H_2DCFDA (**B**) and mROS production measured by mitoSOX Red fluorescence (**C**) at indicated times post-LPS stimulation. Histograms show representative staining. Median fluorescence intensities were normalized to unstimulated WT cells. Graphs show mean ± SEM (n = 3); *p<0.05; **p<0.01; ***p<0.001, two-way ANOVA with Bonferroni correction.

phosphorylation in LPS-activated WT and p21$^{-/-}$ macrophages (Figure 16A), indicating that lack of p21 does not affect the MyD88-independent pathway. We next analyzed phosphorylation of JNK and ERK, both downstream activation indicators of the MyD88-dependent pathway,

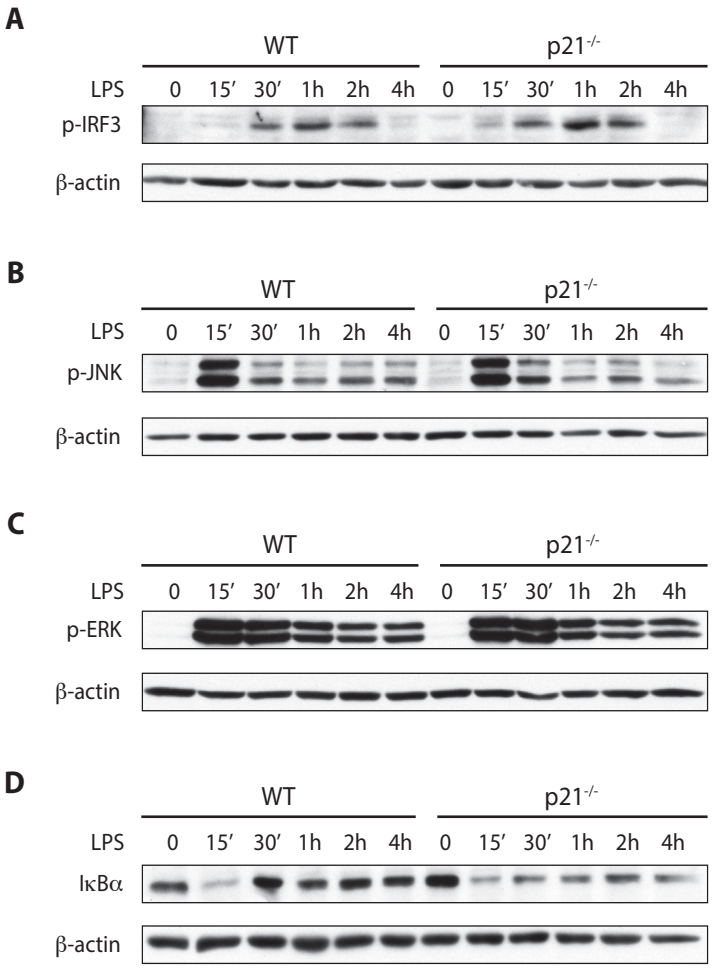


FIGURE 16 | Lack of p21 affects IκBα degradation, but not IRF3 and MAPK pathways in LPS-activated macrophages. Immunoblot analysis shows the phosphorylation of IRF3 (**A**), JNK (**B**), ERK (**C**) and IκBα degradation (**D**) in whole cell lysates of peritoneal macrophages from WT and p21$^{-/-}$ mice stimulated with 100 ng/ml LPS for indicated times. Equal loading is visualized by β-actin expression. Representative results of 2 independent experiments are shown.

and found no differences between WT and p21$^{-/-}$ LPS-activated macrophages (Figure 16B and C). In agreement with a previous study (Trakala et al., 2009), IκBα degradation was more vigorous in p21$^{-/-}$ than in WT macrophages, which is indicative of increased NF-κB activation (Figure 16D). These data show that p21 suppresses exclusively NF-κB activation through negative regulation of IKK complex activity, without affecting the MAPK and MyD88-independent pathways.

p21 deficiency leads to enhanced LPS-induced IFN-γ production in macrophages

We next determined whether p21 deficiency affects the production of TNF-α and IFN-β, end products of the MyD88-dependent and -independent pathways, respectively (Akira and Takeda, 2004). Consistent with previous reports, TNF-α levels were elevated in culture supernatants of LPS-stimulated p21$^{-/-}$ compared with WT macrophages (Figure 17A). We also found increased IFN-β levels in culture supernatants of p21$^{-/-}$ compared with WT macrophages (Figure 17A), in spite of similar IRF3 phosphorylation in p21$^{-/-}$ and WT macrophages. This suggested that the increased IFN-β production is a consequence of increased NF-κB activation in the absence of p21.

LPS-induced IFN-β activates IFNAR (IFN-α/β receptor) in an autocrine manner, leading to STAT1 phosphorylation and transcription of IFN-inducible genes such as iNOS and CXCL11 (Akira and Takeda, 2004). We thus tested whether elevated IFN-β production in the absence of p21 translates into increased activation of IFNAR downstream events. Consistent with previous reports, STAT1 phosphorylation is a late event and was detectable at 1 h post-LPS stimulation (Figure 17B). Although it appears that p-STAT1 is increased in p21$^{-/-}$ compared with WT macrophages at 1 h post-LPS stimulation, p-STAT1 reached saturating levels at the 2 h time point and remained high at 4 h in both WT and p21$^{-/-}$ macrophages (Figure 17B). Coinciding with this finding, iNOS and CXCL11 were strongly induced, with no differences between WT and p21$^{-/-}$ macrophages (Figure 17C). Although p21$^{-/-}$ LPS-activated macrophages produce increased amounts of IFN-β, autocrine IFN-β stimulation in WT cells is sufficient to achieve maximum activation of

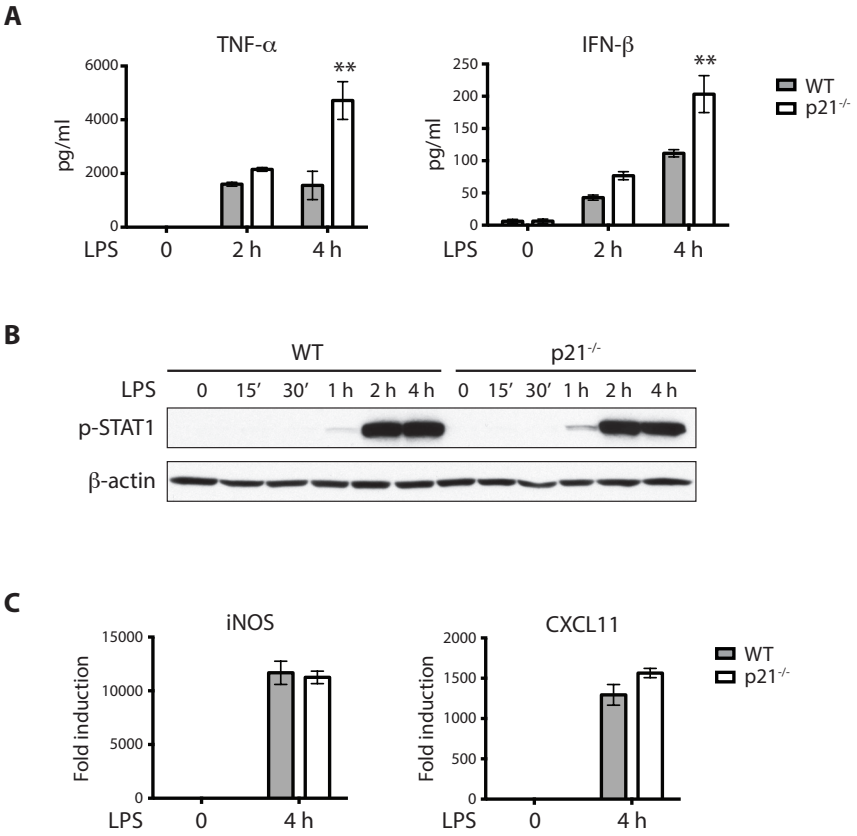


FIGURE 17 | p21 deficiency increases LPS-induced TNF-α and IFN-β production in macrophages. Peritoneal macrophages from WT and p21$^{-/-}$ mice were stimulated with 100 ng/ml LPS for indicated times. (**A**) Cytokine concentrations in culture supernatants at different time points after LPS stimulation, as measured by ELISA. Data show mean ± SEM (n = 3); **p<0.01, two-way ANOVA with Bonferroni correction. (**B**) Immunoblot showing the phosphorylation of STAT1 in whole cell lysates. Equal loading is visualized by β-actin expression. Representative results of 3 independent experiments are shown. (**C**) RT-PCR showing the upregulation of IFN-dependent gene expression. Results were normalized to β-actin and represent fold induction over unstimulated cells. Data show mean ± SEM (n = 3).

IFN-dependent events, at least up to 4 h post-stimulation.

p21 deficiency increases NF-κB DNA binding and promotes the binding of active p65/p50 complex in LPS-activated macrophages

Our data, together with previous studies, suggested that p21 negatively regulates proinflammatory cytokine and IFN-β production through NF-κB. We thus examined the DNA-binding activity of NF-κB in nuclear extracts of WT and p21$^{-/-}$ LPS-activated macrophages, using EMSA. Using a [^{32}P]-labeled consensus probe, we detected notably higher NF-κB DNA binding activity in p21-deficient compared with WT macrophages, at all times post-LPS stimulation (Figure 18A, compare lanes 2 and 5). To determine how NF-κB binding affects IFN-β gene expression, we used the NF-κB element from the IFN-β promoter (Garoufalis et al., 1994), which differs from the consensus NF-κB sequence and serves as a more accurate estimation of the effect of NF-κB activation on IFN-β regulation. Using a [^{32}P]-labeled IFN-β-specific NF-κB probe, we also detected much higher NF-κB DNA binding activity in p21-deficient compared with WT macrophages, at all times post-LPS stimulation (Figure 18B, compare lanes 2 and 5). Control experiments using excess unlabeled or mutant

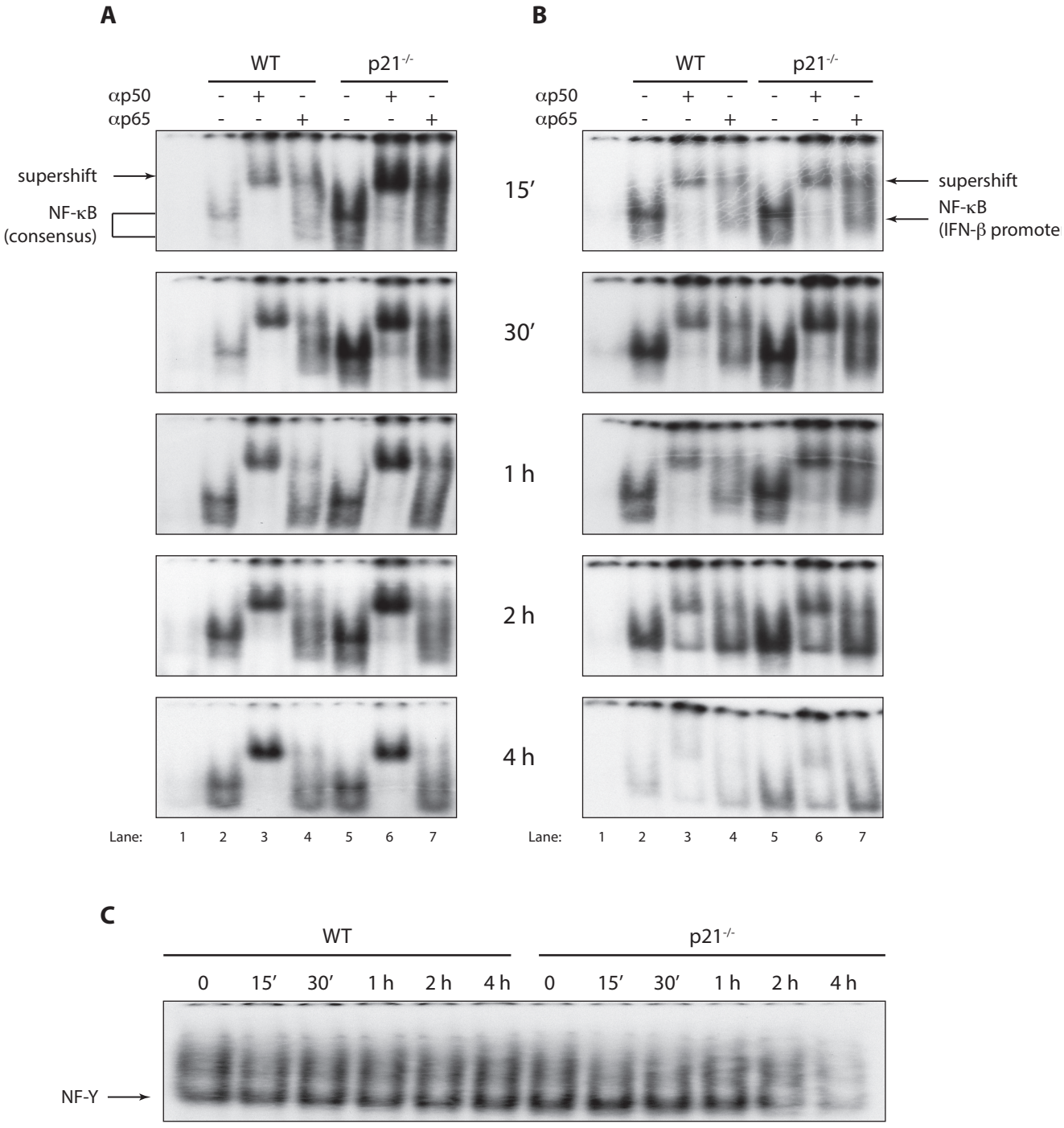


FIGURE 18 | **p21 acts as a negative regulator of NF-κB activity.** Nuclear extracts of WT and p21$^{-/-}$ peritoneal macrophages treated with LPS (100 ng/ml) for indicated times, were incubated with [^{32}P]-labeled NF-κB consensus (**A**) of IFN-β-specific (**B**) probe and NF-κB DNA binding activity was analysed by EMSA. Anti-p65 and -p50 antibodies were used for supershift analysis. (**C**) Equal binding to NF-Y probe was used to verify the quality of nuclear extracts. In all gels, first lane is negative control showing unspecific binding. Representative results of 2 independent experiments are shown.

NF-κB oligonucleotides indicated the specificity of the complexes detected (Figure 18A and B, lane 1).

To examine NF-κB complex composition in WT and p21$^{-/-}$ LPS-activated macrophages, we performed supershift analysis using antibodies specific for p65 and p50 NF-κB subunits. Anti-p50 antibody supershifted the entire NF-κB/DNA complex (Figure 18A and B, lanes 3 and 6), consistent with the fact

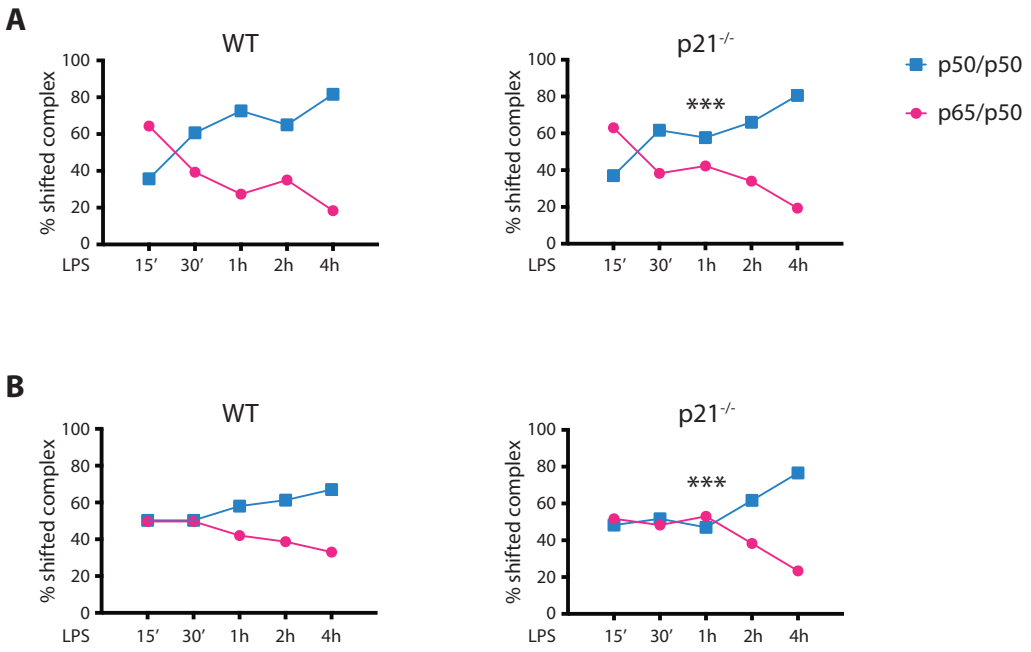


FIGURE 19 | p21 deficiency promotes DNA binding of active p65/p50 NF-κB subunits. Supershift analysis of WT and p21$^{-/-}$ macrophage nuclear extracts was performed using anti-p65 antibody as in Figure 18. The intensity of supershift was assessed by densitometry and plotted as the percent of supershifted complex at indicated time points of LPS stimulation. Graphs show the pattern of p65/p50 binding to consensus (**A**) and IFN-β-specific (**B**) NF-κB sequence in WT and p21$^{-/-}$ macrophages. Data show mean (n = 3 measurements), ***p<0.001, two-way ANOVA. Representative results of 2 independent experiments are shown.

that two most abundant forms of NF-κB in TLR (Toll-like receptor) signaling are active p65/p50 heterodimers and inhibitory p50/p50 homodimers (Kawai and Akira, 2007). By contrast, anti-p65 antibody supershifted only the p65/p50 portion of the DNA/protein complex, while the p50/p50 portion remained unaffected (Figure 18A and B, lanes 4 and 7). This observation allowed the quantification of the relative amounts of p65/p50 *vs.* p50/p50 complexes. At early times post-LPS stimulation, the predominant NF-κB form bound to DNA was active p65/p50 NF-κB, at both the consensus and the IFN-β-specific sequences (Figure 18A and B, lanes 4 and 7). At later times, a shift in the subunit proportion was observed, indicating the predominance of inhibitory p50/p50 NF-κB complexes unaffected by supershift, at both the consensus and the IFN-β-specific sequences (Figure 18A and B, lanes 4 and 7). The NF-κB subunit composition pattern was similar in WT and p21$^{-/-}$ LPS-activated macrophages for both sequences tested, although at 1 h post-stimulation, the absence of p21 favored a larger proportion of active p65/p50 complex, reflecting greater activation potential (Figure 19A and B). During LPS activation, lack of p21 thus increased overall NF-κB activity, as previously shown (Trakala et al., 2009), but also exerted a previously unreported effect in potentiating the predominance of active p65/p50 over the inhibitory p50/50 NF-κB.

Chapter 3: p21 regulates M1 to M2 macrophage reprogramming during endotoxin tolerance through p50/p50 NF-κB

p21 deficiency in mice increases sensitivity to LPS-induced septic shock and death (Trakala et al., 2009). Sepsis is a complex pathology characterized by proinflammatory reactions that are responsible for tissue damage and organ dysfunction in severe sepsis, concurrent with anti-inflammatory responses, which are implicated in leukocyte deactivation, development of endotoxin tolerance and enhanced susceptibility to secondary infections (Angus and van der Poll, 2013; Biswas and Lopez-Collazo, 2009). Endotoxin tolerance in macrophages leads to reprogramming from proinflammatory M1 to hyporesponsive M2-like cells (Porta et al., 2009; Pena et al., 2011). We therefore hypothesized that p21 is involved not only in limiting the proinflammatory responses, but also in promoting the anti-inflammatory response and development of endotoxin tolerance in macrophages.

In vivo role of p21 in endotoxin tolerance

A common approach to the study of endotoxin tolerance in mice is a model that mimics two consecutive pathogen infections (López-Collazo and Del Fresno, 2013). After priming with a sublethal LPS dose, mice develop tolerance and are able to survive subsequent lethal LPS challenge (Berg et al., 1995; Wysocka et al., 2001). We therefore inquired whether a high proinflammatory response in the absence of p21 would compromise LPS tolerance induction. To induce LPS tolerance in WT and p21$^{-/-}$ mice *in vivo*, we administered a sublethal LPS dose (30 μg), followed 16 h later by a lethal LPS dose (150 μg); mice were monitored for 72 h (Figure 20A). We observed a significant difference in survival between WT and p21$^{-/-}$ mice, since the majority of WT mice reached a tolerant state after the secondary LPS challenge, whereas p21$^{-/-}$ mice did not (~60% died by 24 h post-LPS challenge and

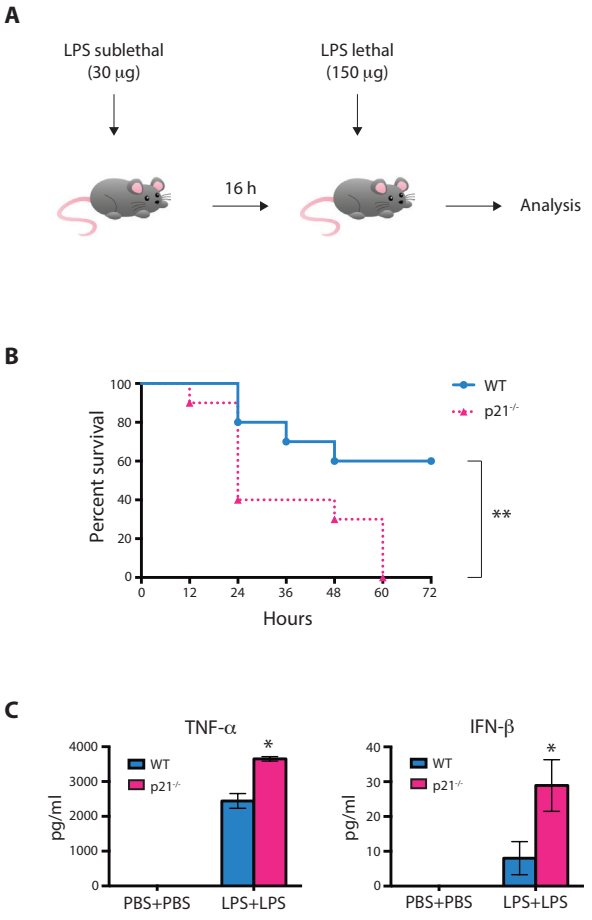


FIGURE 20 | p21 controls *in vivo* endotoxin tolerance. (**A**) WT and p21$^{-/-}$ mice received a sublethal LPS dose, followed by a lethal dose after 16 h. (**B**) p21$^{-/-}$ mice did not develop tolerance compared to WT mice, as represented by a Kaplan-Meier survival curve. *n* = 10 mice; **p<0.01, logrank (Mantel-Cox) test. (**C**) Serum levels of M1-associated cytokines (TNF-α and IFN-β) in WT and p21$^{-/-}$ mice at 2 h post-second LPS injection, as measured by ELISA. Data show mean ± SD (*n* = 3 mice); *p<0.05, two-tailed Student's *t* test.

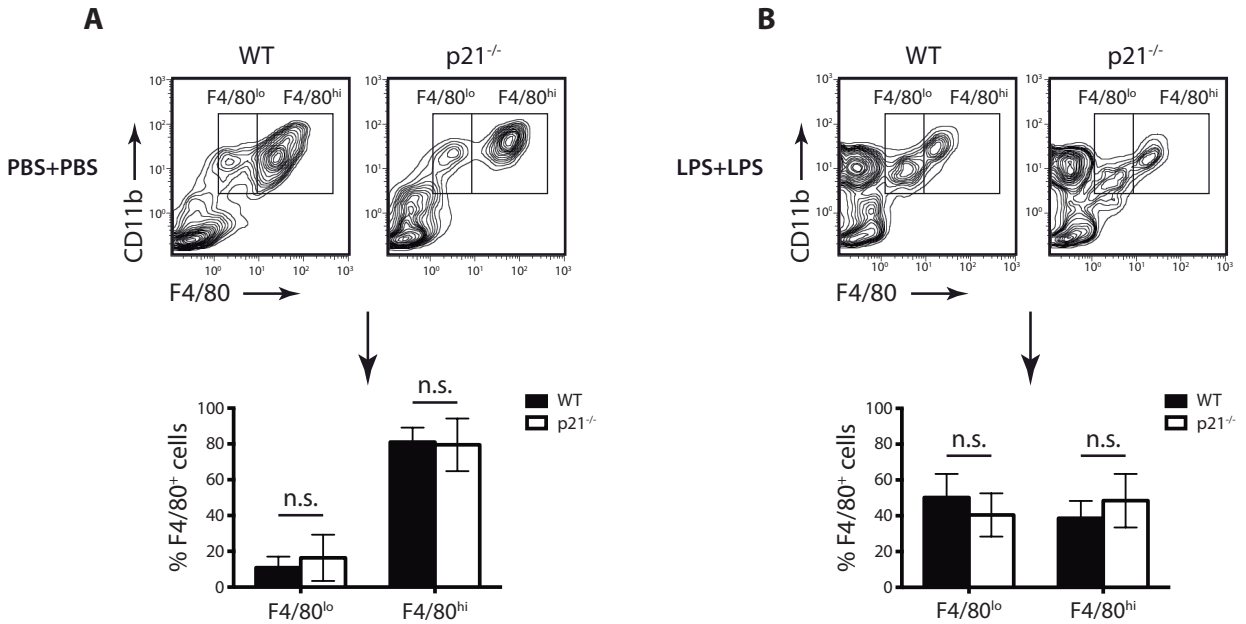


FIGURE 21 | p21 limits M1 activity during *in vivo* endotoxin tolerance. $p21^{-/-}$ and WT mice received two LPS doses as in Figure 20. Flow cytometry analysis of peritoneal macrophage populations at 2 h after dual PBS (**A**) and LPS (**B**) treatment. Representative plots show gated $CD11b^{hi}F4/80^{lo}$ and $CD11b^{hi}F4/80^{hi}$ macrophage populations. The relative percentages of these two populations within the total $F4/80^{+}$ gate are shown below the plots and were similar for WT and $p21^{-/-}$ mice after dual LPS or PBS treatment. Data show mean ± SD (*n* = 8 mice); n.s.-not significant.

100% by 60 h, Figure 20B). For further analysis, we assessed proinflammatory cytokine profiles at 2 h after the second LPS delivery, and found increased serum levels of TNF-α and IFN-β in $p21^{-/-}$ compared with WT mice (Figure 20C). These data indicated that the compromised tolerance is linked to an excess of inflammatory cytokines in $p21^{-/-}$ mice. Based on these results, we hypothesized that p21 is essential for the regulation of macrophage reprogramming from the proinflammatory M1 to the hyporesponsive M2 state.

p21 controls proinflammatory macrophage activity during *in vivo* LPS tolerance

The peritoneal cavity of unstimulated mice is characterized by the presence of $CD11b^{+}F4/80^{hi}$ macrophages, also referred to as large peritoneal macrophages; i.p. LPS injection provokes an influx of functionally distinct $CD11b^{+}F4/80^{lo}$ cells, called small peritoneal macrophages (Ghosn et al., 2010; Ní Gabhann et al., 2014; Singh et al., 2015). We tested whether lack of p21 affects these macrophage populations during *in vivo* induction of LPS tolerance.

The relative proportions of peritoneal $CD11b^{+}F4/80^{lo}$ and $CD11b^{+}F4/80^{hi}$ were similar in WT and $p21^{-/-}$ mice after dual PBS treatment, showing a preponderance of $F4/80^{hi}$ with a $F4/80^{lo}$:$F4/80^{hi}$ ratio of ~10:90 (Figure 21A). After dual LPS treatment, this ratio changed to ~50:50 in both WT and $p21^{-/-}$ mice (Figure 21B), reflecting no apparent differences in the macrophage populations in WT and $p21^{-/-}$ mice during LPS tolerance. Analysis of the activation state of these populations nonetheless showed a >2-fold increase in TNF-α production in $p21^{-/-}$ compared with WT $F4/80^{lo}$ macrophages, indicating a greatly elevated M1-like macrophage response in the absence of p21 (Figure 22A). In agreement with this, we detected markedly augmented expression of

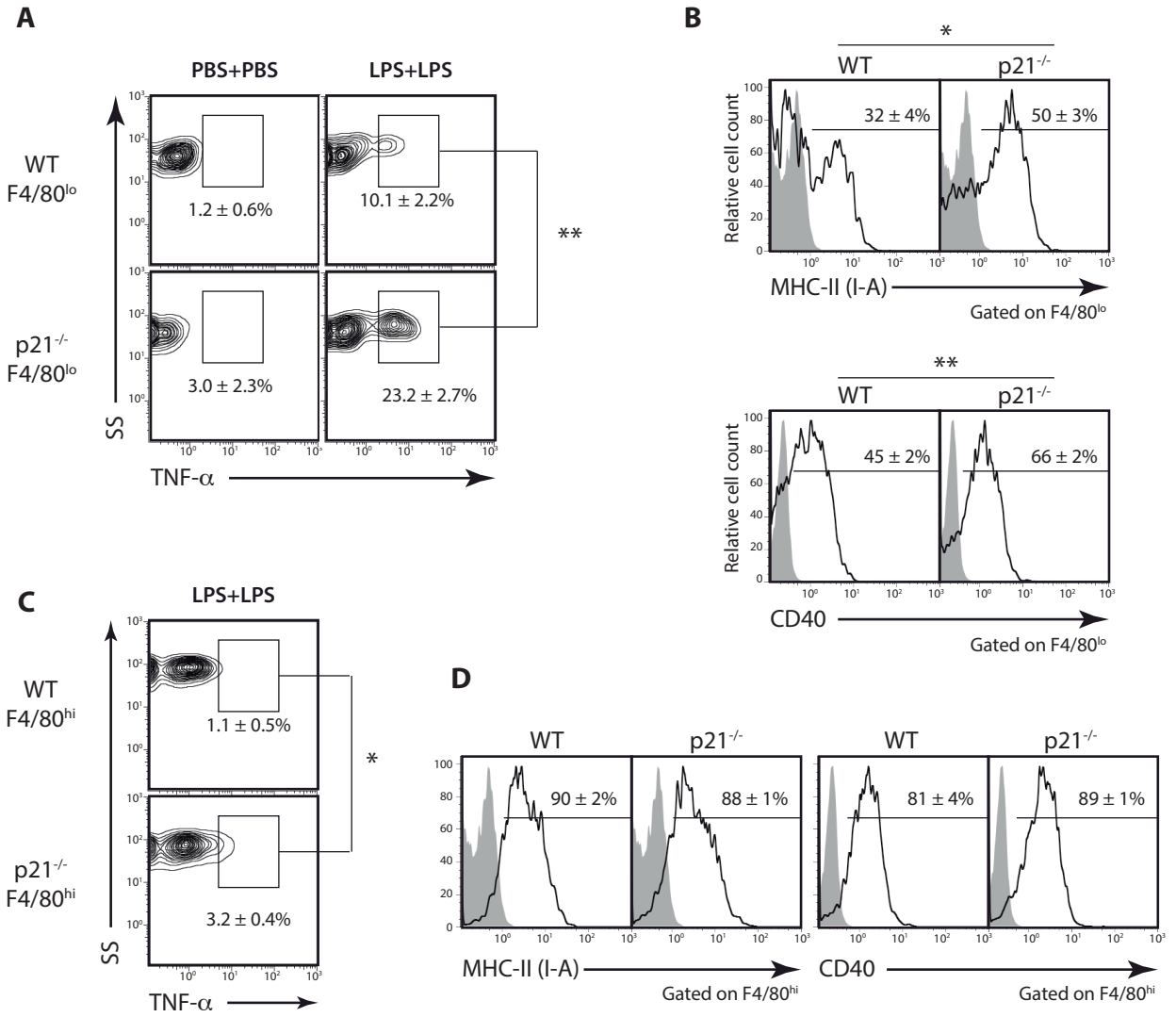


FIGURE 22 | p21 limits proinflammatory activity during *in vivo* endotoxin tolerance. p21$^{-/-}$ and WT mice received two LPS doses as in Figure 20. (**A**) Intracellular staining showing TNF-α production and by the F4/80^{lo} macrophage population at 2 h post-treatment. (**B**) Expression of activation markers MHCII and CD40 on cell surface of F4/80^{lo} macrophage population. Intracellular TNF-α **(C)** and extracellular MHCII and CD40 (**D**) levels in F4/80^{hi} macrophage population. Data show mean ± SEM (*n* = 3 mice); *p<0.05; **p<0.01, two-tailed Student's *t* test.

MHC (major histocompatibility complex) class II and CD40 activation markers in p21$^{-/-}$ compared with WT F4/80^{lo} macrophages (Figure 22B). Although F4/80^{hi} macrophages produced low TNF-α levels, lack of p21 increased the production of this cytokine (Figure 22C). MHC class II and CD40 markers were expressed strongly in WT F4/80^{hi} macrophages (>80%) and did not increase further in the absence of p21 (Figure 22D). Collectively, these data show that although lack of p21 does not alter the relative proportions of peritoneal macrophage populations, it has a profound effect on their activation and M1 proinflammatory state. During *in vivo* LPS tolerization, the increased proinflammatory state of macrophages in p21$^{-/-}$ mice thus appears to impair the development of tolerance and leads to death.

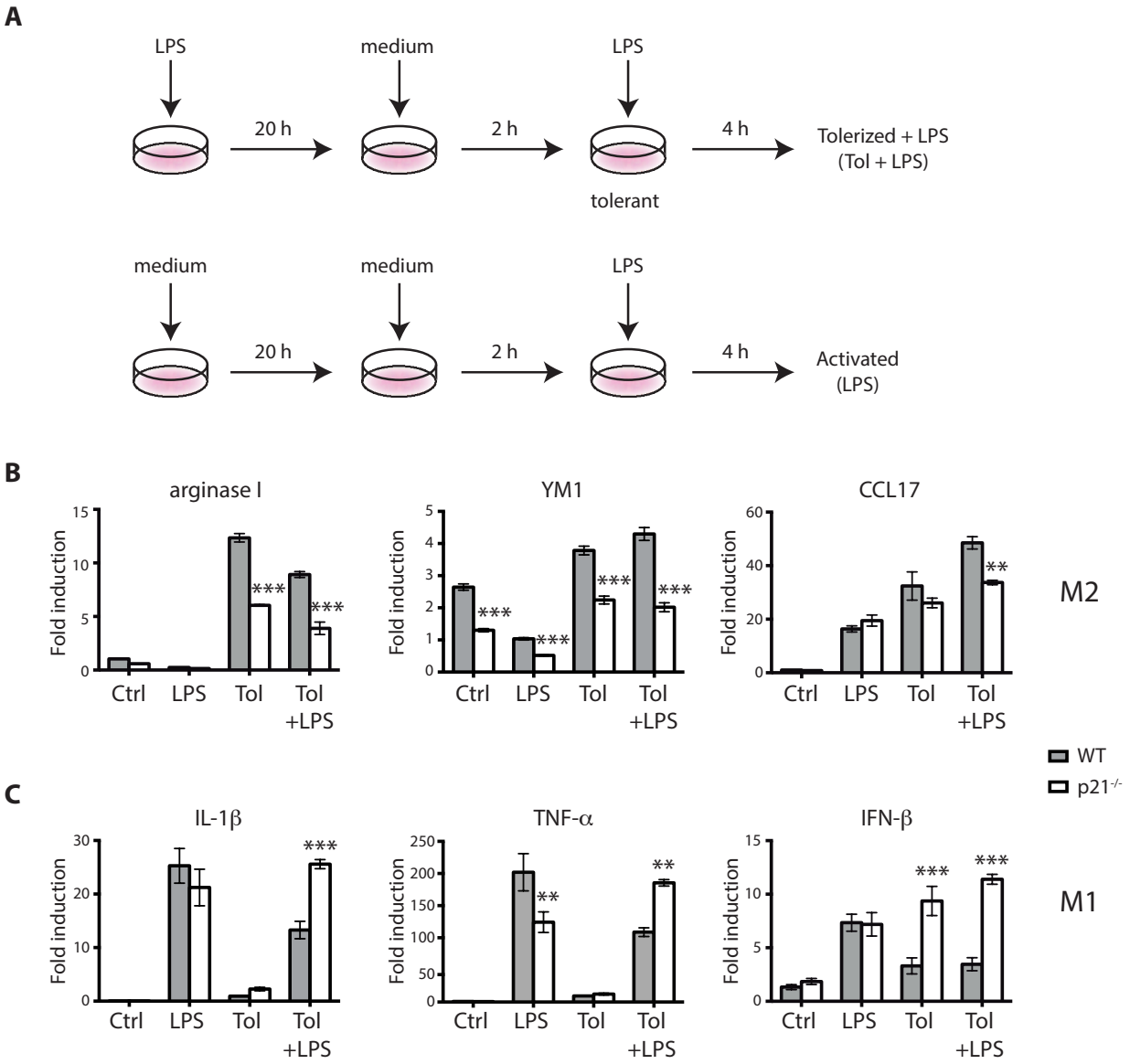


FIGURE 23| p21$^{-/-}$ macrophages show impaired ability to polarize to M2 cells during *in vitro* endotoxin tolerance. (**A**) Scheme showing the *in vitro* endotoxin tolerance model. Peritoneal macrophages from WT and p21$^{-/-}$ mice were tolerized with 100 ng/ml LPS for 20 h (Tol), washed with PBS, cultured in medium (2 h) and restimulated with 100 ng/ml LPS for 4 h (Tol + LPS). LPS-activated cells were stimulated with LPS for 4 h without previous tolerization (LPS). Cells left untreated were controls (Ctrl). Total RNA was extracted at 4 h after LPS treatment and analyzed for gene expression. Culture supernatants were also collected and analyzed for cytokine production. (**B**) RT-PCR analysis showed impaired upregulation of M2-associated genes in p21$^{-/-}$ compared with WT macrophages after tolerization. (**C**) RT-PCR showed upregulation of representative M1 cytokine genes in p21$^{-/-}$ compared with WT tolerized macrophages. Results were normalized to β-actin and represent fold induction over unstimulated WT cells. In all cases data show mean ± SEM (*n* = 3 independent experiments), **p*<0.05; ***p*<0.01; ****p*<0.001, two-way ANOVA with Bonferroni correction.

p21 deficiency disrupts M1 to M2 macrophage polarization during LPS tolerance *in vitro*

Our *in vivo* results suggested that lack of p21 might promote M1 macrophage responses and interfere with M2 polarization during endotoxin tolerance. To examine this possibility, we studied the role of p21 in macrophage polarization during *in vitro* LPS tolerance. In macrophages, tolerance is induced by prolonged LPS treatment, which leads to acquisition of an M2 phenotype and reduced responsiveness of M1-associated genes after a second LPS challenge (Porta et al., 2009). To induce tolerance *in vitro*, murine peritoneal macrophages were treated

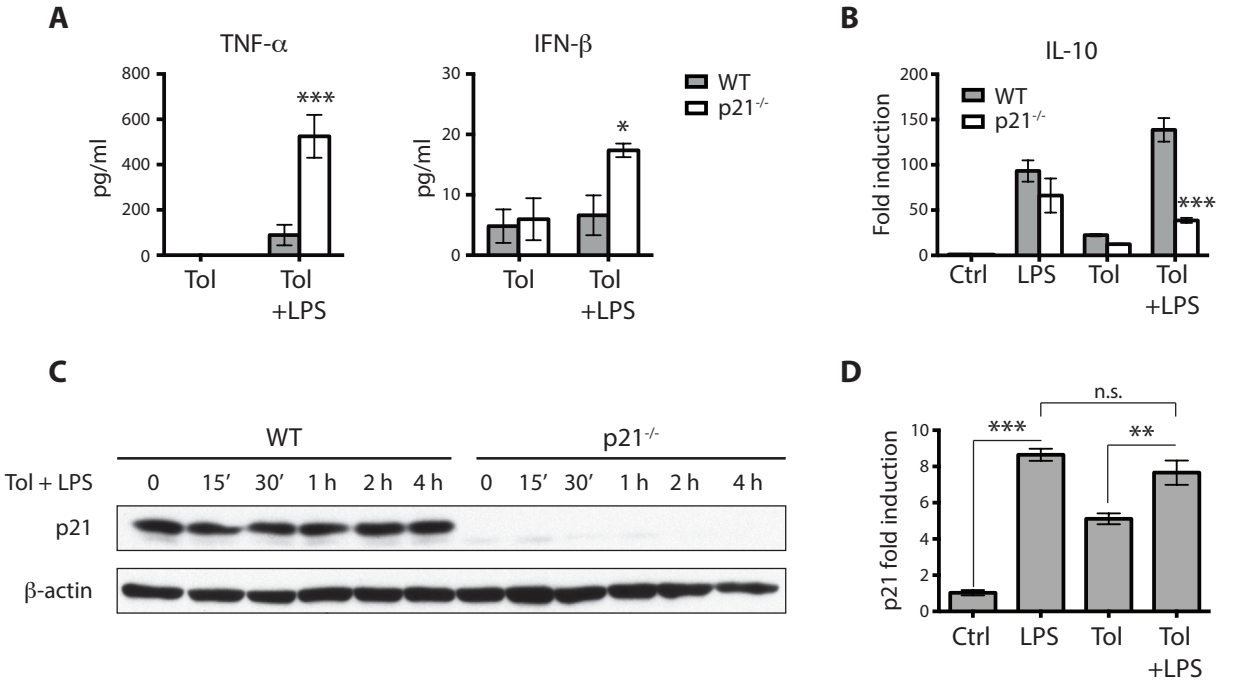


FIGURE 24 | p21 deficiency increases TNF-α and IFN-β production decreases IL-10 in LPS-tolerized macrophages. Peritoneal macrophages from WT and p21$^{-/-}$ mice were tolerized as in Figure 23. (**A**) M1-associated cytokine production after LPS-restimulation, as measured by ELISA. (**B**) After LPS tolerization, p21$^{-/-}$ macrophages showed decreased IL-10 induction compared with WT macrophages. Results were normalized to β-actin and represent fold induction over unstimulated cells of each genotype. p21 gene expression, as seen by Western blot (**C**) and by RT-PCR (**D**) was induced to similar levels in LPS-activated and -tolerized macrophages. Results were normalized to β-actin and represent fold induction over unstimulated WT cells. In all cases data show mean ± SEM (n = 3 independent experiments), *p<0.05; **p<0.01; ***p<0.001.

with LPS (20 h), washed, allowed to rest (2 h) and restimulated with LPS (4 h) (Porta et al., 2009) (Figure 23A). This treatment was followed by RT-PCR analysis of prototypical M1 and M2 gene expression in unstimulated (Ctrl), activated (LPS), tolerized (Tol) and restimulated (Tol + LPS) macrophages. Whereas tolerant WT macrophages acquired an M2 polarization profile characterized by high expression of prototypic M2 genes (arginase I, YM1, CCL17), expression of these genes was defective in p21$^{-/-}$ macrophages (Figure 23B). These results revealed a central role for p21 in M1 to M2 macrophage reprogramming. In support of this view, WT macrophages were tolerized as reflected by downregulation of M1 genes (IL-1β, TNF-α, IFN-β), whereas p21$^{-/-}$ macrophages did not develop tolerance and showed significantly higher levels of M1-associated cytokine expression after LPS rechallenge (Figure 23C). IFN-β mRNA was upregulated in tolerized p21$^{-/-}$ macrophages even without LPS restimulation (Figure 23C), suggesting close linkage of p21 and IFN-β in the regulation of M1 to M2 macrophage reprogramming.

In culture supernatants of LPS-restimulated macrophages, levels of M1 cytokines TNF-α and IFN-β were significantly elevated in the absence of p21 (Figure 24A), which further confirmed the role of p21 in the control of macrophage tolerization. We also evaluated expression of IL-10, an M2-related cytokine that is upregulated during endotoxin tolerance (Porta et al., 2009), and found impaired IL-10 induction in p21$^{-/-}$ macrophages (Figure 24B). In accordance with the role of p21 in M2

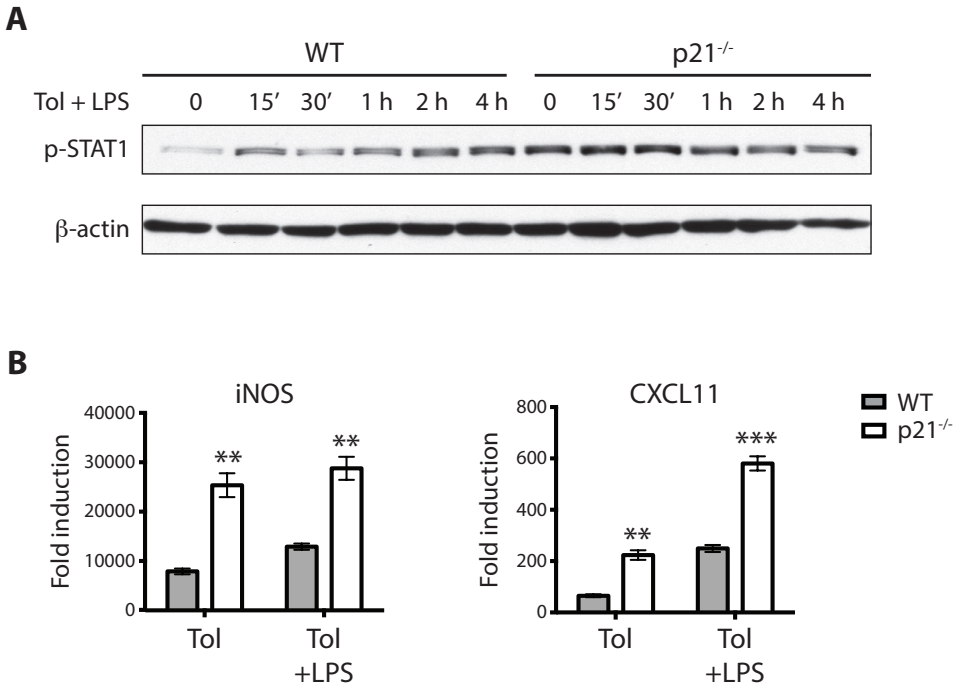


FIGURE 25 | Increased IFN-β production disrupts LPS tolerization in p21-deficient macrophages. (**A**) Immunoblot showing STAT1 phosphorylation after LPS re-stimulation of tolerized WT and p21$^{-/-}$ macrophages. β-actin was used as a loading control. (**B**) RT-PCR showing increased induction of iNOS and CXCL11 in tolerized p21$^{-/-}$ macrophages as compared with WT. Results were normalized to β-actin and show fold induction over unstimulated WT cells. Data shown as mean ± SEM (*n* = 3 independent experiments); ***p*<0.01; ****p*<0.001, two-way ANOVA with Bonferroni correction.

polarization, we detected high p21 protein levels at early time points and up to 4 h post-LPS restimulation (Figure 24C); p21 mRNA was significantly induced after LPS rechallenge and reached levels similar to those observed in LPS-activated cells (Figure 24D). It thus appears that p21 promotes M1 to M2 polarization during *in vitro* endotoxin tolerization, and that IFN-β regulation is essential in the control of this process.

Increased IFN-β production disrupts LPS tolerization in p21-deficient macrophages

Our data showed that lack of p21 leads to increased IFN-β production after LPS activation (Figure 17A), although these high IFN-β levels had no effect on downstream gene expression after 4 h of LPS stimulation (Figure 17B and C). Sustained IFN-β production might nonetheless disrupt the tolerization process after prolonged LPS exposure, by maintaining strong expression of IFN-β-dependent M1-associated genes such as iNOS and CXCL11 (Akira and Takeda, 2004). To provide a cause/effect confirmation for the role of p21 on IFN-β regulation during LPS tolerance, we examined whether lack of p21 promotes IFN-dependent events, such as STAT1 phosphorylation (p-STAT1) and M1-associated gene expression. Following LPS tolerization (time 0; Figure 25A), WT cells showed low p-STAT1 levels, indicative of effective tolerization, while p21$^{-/-}$ macrophages showed high p-STAT1 levels and resisted tolerization. After secondary LPS treatment, STAT1 phosphorylation remained considerably higher in p21-deficient than in WT macrophages at 15, 30, and 60 min (Figure 25A). The association of p21 with p-STAT1 regulation during tolerization was further supported, since lack of p21 led to significantly higher iNOS and CXCL11 levels after LPS tolerization and restimulation (Figure 25B). These results support the hypothesis that p21 is critical for

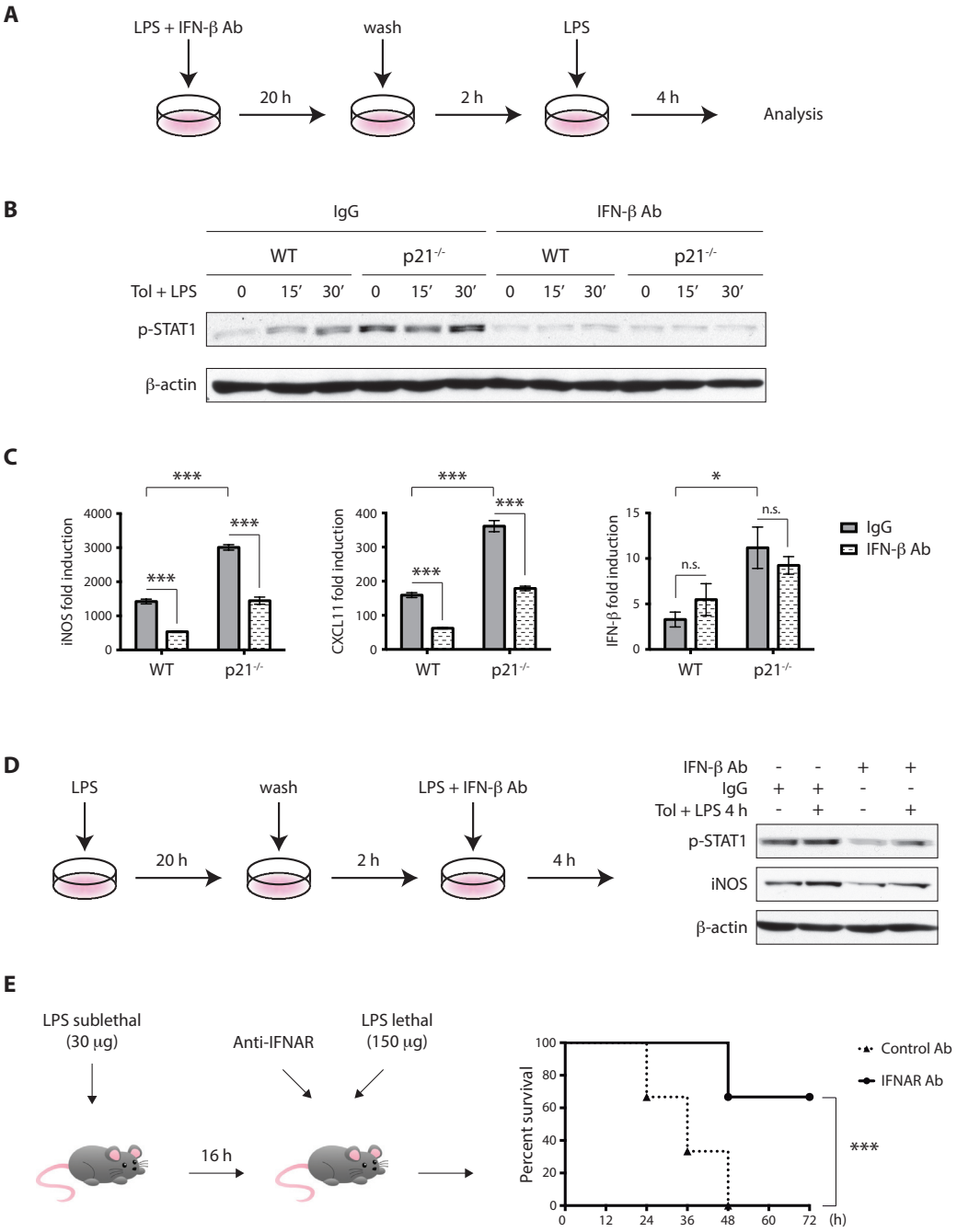


FIGURE 26 | IFN-β neutralization reduces STAT1 phosphorylation and induces hyporesponsiveness development in p21$^{-/-}$ macrophages. (**A**) WT and p21$^{-/-}$ peritoneal macrophages were incubated with an IFN-β-neutralizing or an isotype control antibody during LPS tolerization (20 h). Cells were washed, cultured in medium (2 h) and restimulated with LPS (4 h). (**B**) Immunoblot showing reduced STAT1 phosphorylation after antibody treatment. (**C**) RT-PCR analysis showing iNOS and CXCL11 expression in WT and p21$^{-/-}$ macrophages treated with IFN-β-neutralizing antibody compared with isotype control-treated cells. Results were normalized to β-actin and show fold induction over unstimulated WT cells (not shown). Data shown as mean ± SEM (n = 3 independent experiments); *p<0.05; **p<0.01; ***p<0.001. (**D**) After LPS tolerization (20 h) and incubation in medium (2 h), p21$^{-/-}$ peritoneal macrophages were LPS-restimulated (4 h) in the presence of the IFN-β-neutralizing or an isotype control antibody. Immunoblot showing reduced STAT1 phosphorylation and iNOS expression after antibody treatment. (**E**) p21$^{-/-}$ mice were challenged with two LPS doses as in Figure 20. Two hours before LPS rechallenge, mice were treated i.p. with either anti-IFNAR1 monoclonal antibody or control IgG. Inhibition of IFNAR1 significantly improved tolerance to LPS, as compared with the control group and represented by a Kaplan-Meier survival curve. n = 9 mice per group; ***p<0.001, log rank (Mantel-Cox) test. In all immunoblotting analyses β-actin was used as a loading control.

induction of macrophage hyporesponsiveness, by regulating IFN-β-dependent STAT1 activation. This view was reinforced by the elevated IFN-β production by p21$^{-/-}$ compared to WT macrophages (Figure 24A).

IFN-γ neutralization promotes macrophage hyporesponsiveness and improves LPS tolerance in p21$^{-/-}$ mice

To provide direct evidence that lack of p21 enhances IFN-β production and promotes STAT1 phosphorylation, we treated WT and p21$^{-/-}$ macrophage cultures with an IFN-β-blocking antibody (or an isotype-matched control). Antibody treatment lasted for the 20 h of LPS tolerization, after which cells were washed and LPS rechallenged. This antibody treatment efficiently reduced STAT1 phosphorylation in p21$^{-/-}$ cells, resulting in similar p-STAT1 levels in WT and p21$^{-/-}$ cells after tolerization (time 0; Figure 26A) and after secondary LPS treatment (Figure 26A). Anti-IFN-β antibody treatment effectively reduced iNOS and CXCL11 expression in p21$^{-/-}$ restimulated macrophages, which reverted to the levels produced by WT cells, and also suppressed the expression of these genes in WT cells (Figure 26B). We detected no effect on endogenous IFN-β expression (Figure 26B). These results showed that IFN-β neutralization reduces M1 activity mediators in p21$^{-/-}$ macrophages during tolerization, and coincide with the view that elevated IFN-β production hinders the macrophage potential to attain a hyporesponsive state.

To determine whether the effects of elevated IFN-β production by p21$^{-/-}$ macrophages extended beyond the 20 h tolerization time frame, we initiated IFN-β neutralization treatment after tolerization. p21-deficient macrophages were tolerized with LPS

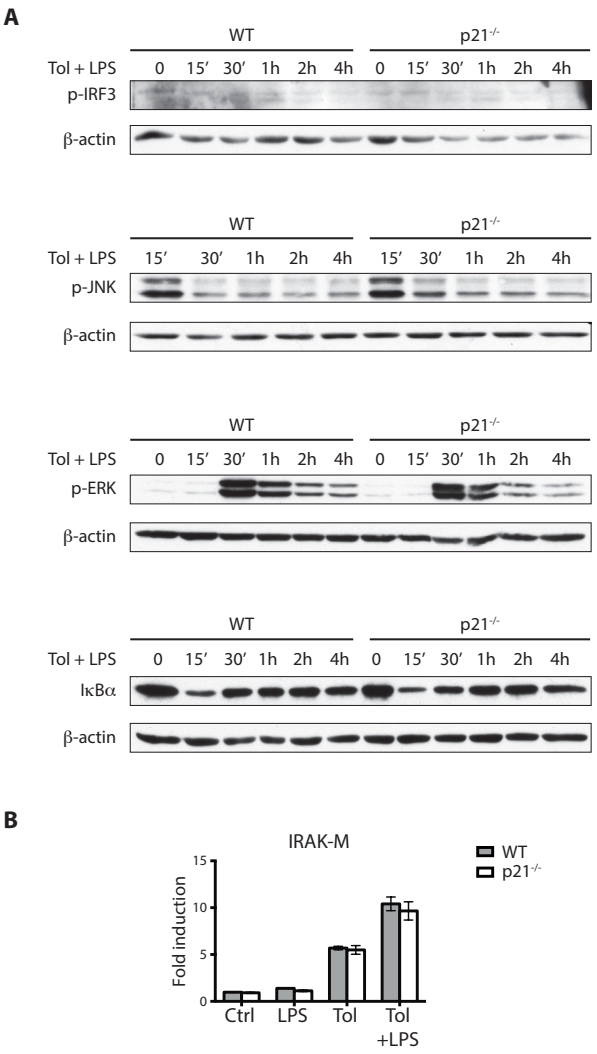


FIGURE 27 | **Lack of p21 does not affect IRF3, MAPK and NF-κB pathways in LPS-tolerized macrophages.** (**A**) Immunoblots showing IRF3, ERK and JNK phosphorylation, and IκBα degradation at indicated times after LPS stimulation of resting or tolerized macrophages. β-actin is included as a loading control. Shown is representative experiment of two performed. (**B**) IRAK-M was induced at similar levels in LPS-tolerized WT and p21$^{-/-}$ macrophages. Results were normalized to β-actin and represent fold induction over unstimulated WT cells. Data shown as mean ± SEM (*n* = 3).

(20 h), subsequently treated with an anti-IFN-β antibody, and LPS rechallenged. Antibody treatment reduced STAT1 phosphorylation and iNOS protein levels (Figure 26C). This indicates that IFN-β neutralization reverses the M1 proinflammatory phenotype of p21$^{-/-}$ macrophages to a tolerant state, even when antibody is delivered after LPS tolerization, during LPS rechallenge. These data suggest

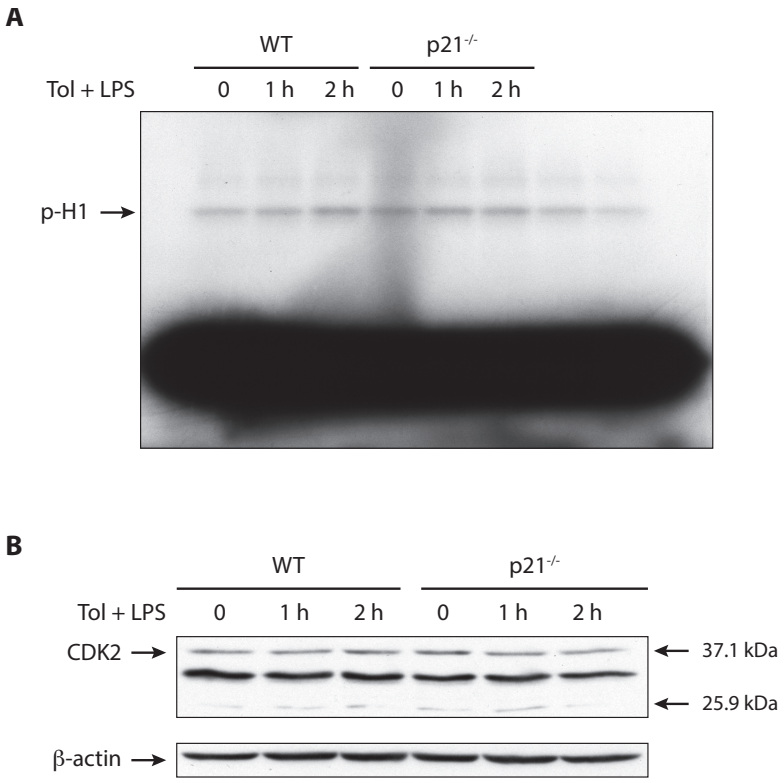


FIGURE 28 | Impaired LPS tolerance in p21$^{-/-}$ macrophages is cell-cycle-independent. (**A**) In WT and p21$^{-/-}$ LPS-tolerized macrophage extracts, CDK2 was immunoprecipitated and its kinase activity was measured using histone H1 as a substrate. Assay products were resolved in SDS-PAGE, and phosphorylated H1 was detected by autoradiography. (**B**) Immunoblot showing CDK2 protein levels in LPS-tolerized peritoneal macrophages. Equal loading is visualized by β-actin expression. Representative results of 2 independent experiments are shown.

that elevated IFN-β is a determinant factor in compromising p21$^{-/-}$ macrophage progression to hyporesponsiveness, and acts at all levels of the tolerization process.

To confirm that IFN-β impairs macrophage hyporesponsiveness *in vivo*, p21$^{-/-}$ mice, which fail to develop tolerance after two consecutive LPS challenges, were treated with anti-IFNAR (interferon-α/β receptor) antibody 2 h before secondary LPS delivery. In accordance with our findings showing that IFN-β neutralization promotes the *in vitro* tolerization of p21$^{-/-}$ macrophages, *in vivo* anti-IFNAR treatment induced tolerance and significantly improved survival of p21$^{-/-}$ mice after dual LPS injection (Figure 26D). Our data support the idea that the inability of p21$^{-/-}$ macrophages to achieve the hyporesponsive state is directly associated to the potential of these cells to produce high IFN-β levels, and point to IFN-β as a major factor in disabling macrophage tolerization.

Lack of p21 does not affect IRF3, MAPK and NF-κB pathways in LPS-tolerized macrophages

To understand how lack of p21 mediates increased IFN-β production after repeated LPS stimulation in macrophages, we examined activation pathways associated with this cytokine. IFN-β expression can be activated in a MyD88-independent or -dependent manner, through IRF3 or NF-κB, respectively (Akira and Takeda, 2004). Analysis of the MyD88-independent pathway showed no detectable change in IRF3 phosphorylation in LPS-tolerized and -restimulated WT or p21$^{-/-}$ macrophages (Figure 27A), suggesting that lack of p21 does not affect this pathway. We next analyzed downstream activation indicators of the MyD88-dependent pathway such ERK and JNK phosphorylation and found no differences after secondary LPS challenge in WT and p21$^{-/-}$ cells (Figure 27A). As p21 deficiency leads to increased IκBα degradation (Figure 16D) and increased NF-κB activity after primary LPS treatment (Figure 18), we examined the degree of IκBα degradation after LPS treatment in

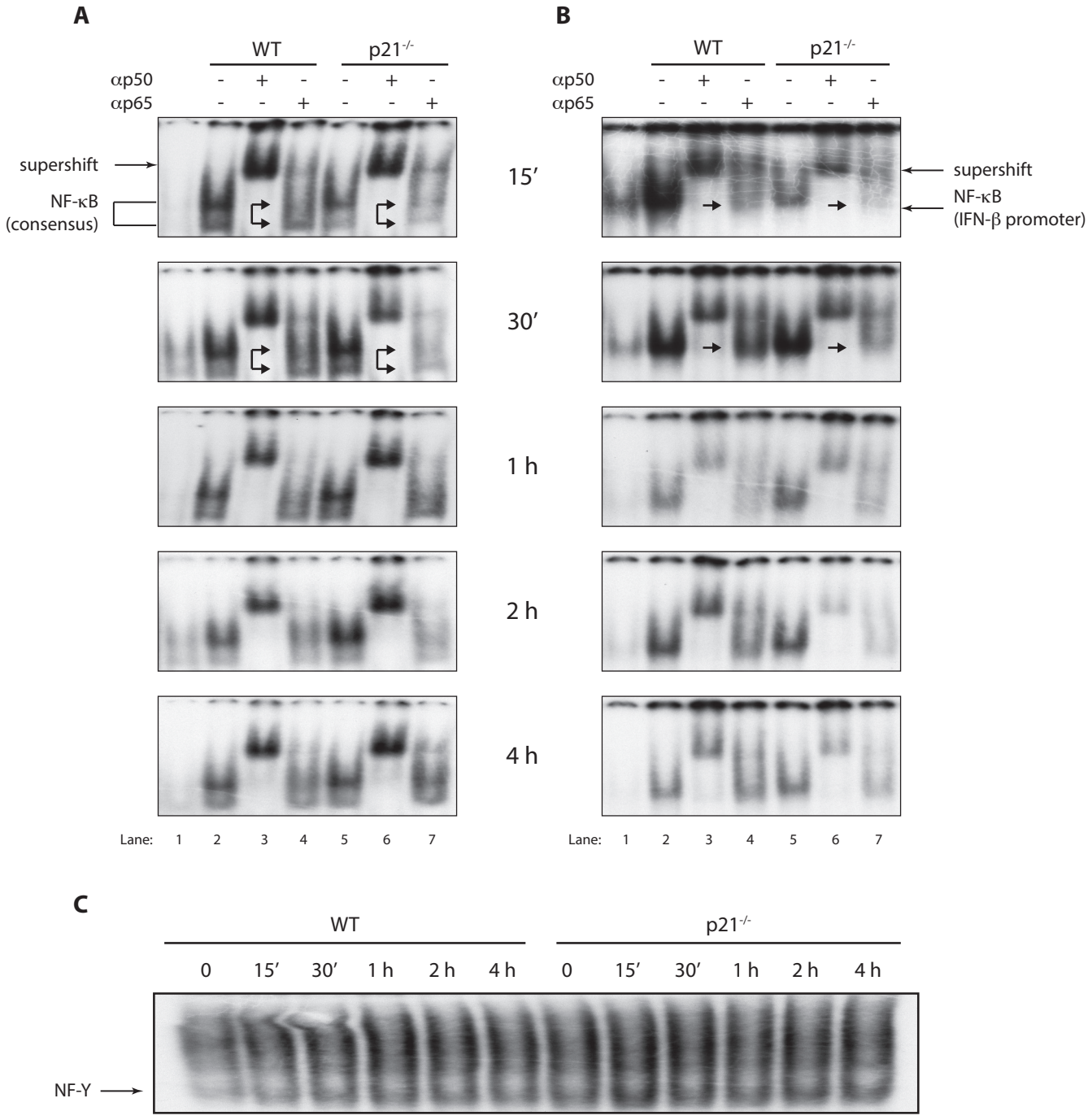


FIGURE 29 | p21 deficiency does not increase the overall NF-κB DNA binding activity in LPS tolerized macrophages. WT and p21$^{-/-}$ peritoneal LPS-tolerized macrophages were restimulated with LPS (100 ng/ml) for indicated times, and their nuclear extracts were incubated with [^{32}P]-labeled NF-κB consensus (**A**) of IFN-β-specific (**B**) probe and NF-κB DNA binding activity was analysed by EMSA. Anti-p65 and -p50 antibodies were used for supershift analysis. (**C**) Equal binding to NF-Y probe was used to verify the quality of nuclear extracts. In all gels, first lane is negative control showing unspecific binding. Representative results of 2 independent experiments are shown.

tolerized macrophages. IκBα levels were similar in WT and p21$^{-/-}$ macrophages following LPS rechallenge, which indicated that lack of p21 does not affect the NF-κB pathway in LPS-tolerized macrophages (Figure 27A). As IRAK-M is an important negative regulator of the TLR4 pathway that is implicated in LPS tolerance (Kobayashi et al., 2002), we examined whether p21 deficiency affected this regulator. We found no differences in IRAK-M expression

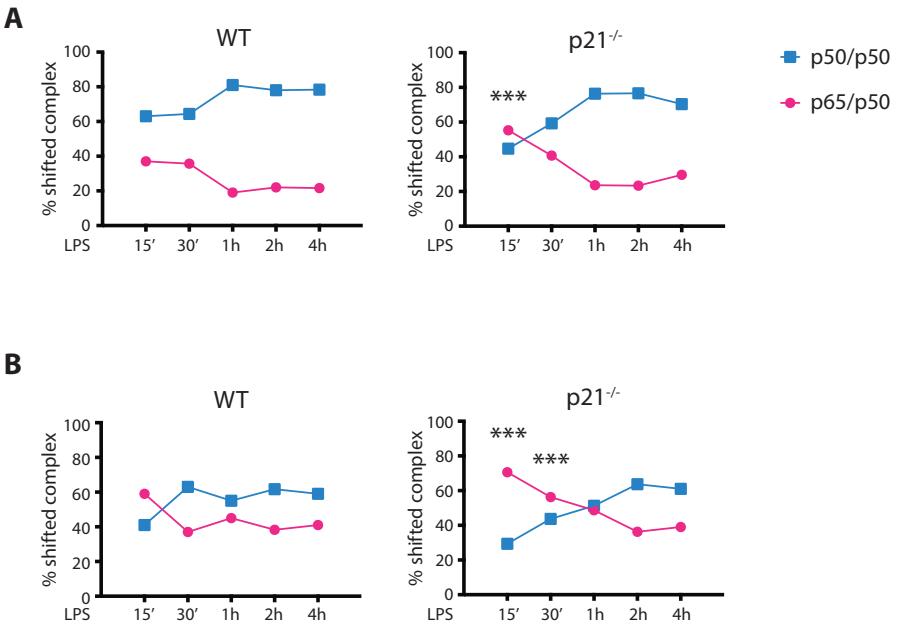


FIGURE 30 | p21 deficiency modulates the balance between p65/p50 and p50/p50 NF-κB dimers in LPS tolerized macrophages. Supershift analysis of WT and p21$^{-/-}$ macrophage nuclear extracts was performed using anti-p65 antibody as in Figure 29. The intensity of supershift was assessed by densitometry and plotted as the percent of supershifted complex at indicated time points of LPS stimulation. Graphs show the pattern of p65/p50 *vs.* p50/p50 binding to consensus (**A**) and IFN-β-specific (**B**) NF-κB sequence in WT and p21$^{-/-}$ macrophages. Data show mean (n = 3 measurements), ***p<0.001, two-way ANOVA. Representative results of 2 independent experiments are shown.

between WT and p21$^{-/-}$ macrophages after LPS tolerization and rechallenge (Figure 27B).

Since LPS treatment in macrophages induces cell cycle arrest (Xaus et al., 1999) and p21 is cell cycle inhibitor, we evaluated whether p21 deficiency affects the cell cycle after LPS tolerization. We examined activity and expression of CDK2, a p21 target, and found that CDK2 activity in LPS-tolerized macrophages was independent of p21 expression (Figure 28A). Moreover, CDK2 protein levels were similar in WT and p21$^{-/-}$ macrophages at all times post-LPS restimulation (Figure 28B). p21 deficiency thus did not affect cell cycling in LPS-tolerized macrophages.

p21 attenuates IFN-β expression by regulating the balance of p50/p50 and p65/p50 NF-κB

The fact that lack of p21 leads to enhanced NF-κB activity after LPS activation (Figure 18) led us to hypothesize that it would have a similar effect in LPS-tolerized cells. To test this possibility, we examined DNA-binding activity of NF-κB in nuclear extracts of WT and p21$^{-/-}$ LPS-rechallenged macrophages by EMSA using a [^{32}P]-labeled consensus NF-κB probe. We found that NF-κB activation was similar in WT and p21$^{-/-}$ LPS-tolerized macrophages at all time points following secondary stimulation (Figure 29A, compare lanes 2 and 5). As our data showed that IFN-β levels are determinant for the increased proinflammatory response of p21$^{-/-}$ macrophages after LPS tolerization and rechallenge (Figure 26), we analyzed how NF-κB activation affects IFN-β gene expression, using the NF-κB element from the IFN-β promoter (Garoufalis et al., 1994). Overall

NF-κB binding to the IFN-β-specific sequence did not increase in p21$^{-/-}$ macrophages after LPS rechallenge (Figure 29B, compare lanes 2 and 5), suggesting that p21 acts through two different mechanisms in LPS-activated and tolerized macrophages.

Following LPS tolerization, inhibitory p50 NF-κB accumulates and forms homodimers that repress transcription of NF-κB target genes (Ziegler-Heitbrock, 2001). We hypothesized that lack of p21 could affect the balance of active p65/p50 and inhibitory p50/50 NF-κB complexes in favor of p65/50, and thus disrupt macrophage polarization and development of tolerance. To examine the NF-κB complex composition in WT and p21$^{-/-}$ LPS-rechallenged macrophages, we performed supershift analysis using antibodies specific for p65 and p50 NF-κB subunits. Anti-p50 antibody supershifted the entire complex (Figure 29A and B, lanes 3 and 6), consistent with the fact that p65/p50 and p50/p50 forms of NF-κB are most abundant in this system (Kawai and Akira, 2007). By contrast, anti-p65 antibody supershifted only the p65/p50 portion of the DNA-protein complex, leaving the p50/p50 portion unaffected; this permitted quantification of the relative amounts of p65/p50 *vs.* p50/p50 complexes (Figure 29A and B, lanes 4 and 7). At all times post-LPS rechallenge, the predominant NF-κB form bound to consensus sequence in WT macrophages was inhibitory p50/p50 (Figure 29A, lane 4 and Figure 30A), which corroborated the compromised inflammatory status of tolerized macrophages. In the case of p21$^{-/-}$ macrophages, the profile of NF-κB complex composition differed, and showed higher levels of p65/p50 over p50/p50 complexes at 15 min post-secondary stimulation (Figure 29A, lane 7 and Figure 30A). At later times, however, the relative proportion of these complexes reverted to that of WT macrophages (Figure 29A, lane 7 and Figure 30A). These findings suggested that the inability of p21$^{-/-}$ macrophages to achieve a tolerant phenotype after secondary LPS stimulation is linked to altered balance of NF-κB complexes and delayed formation of inhibitory p50/p50 NF-κB in these cells.

Analysis of our data using an IFN-β-specific NF-κB probe showed a striking difference in NF-κB complex composition, with decreased proportions of p50/p50 homodimers in p21$^{-/-}$ compared to WT macrophages at 15 and 30 min post-LPS rechallenge (Figure 29B, lanes 4 and 7 and Figure 30B). We observed the most evident difference at 30 min post-LPS rechallenge, with a clear predominance of p50/p50 homodimers in WT macrophages, while p65/p50 heterodimers were found in much larger proportions in p21$^{-/-}$ cells (Figure 29B, lanes 4 and 7 and Figure 30B). The NF-κB subunit-binding pattern was much more affected by the lack of p21 in the case of the IFN-β promoter-specific sequence than in the case of the consensus probe (Figure 30 A and B). This difference might be due to the distinct affinities of p65/p50 and p50/p50 NF-κB for the two NF-κB-binding sequences (Frankenberger and Löms Ziegler-Heitbrock, 1997).

Overall, these results show that lack of p21 affects the degree of NF-κB activation in macrophages after primary, but not after secondary LPS treatment. Absence of p21 after secondary stimulation disrupts the accumulation of p50/p50 homodimers that limit the proinflammatory responses of macrophages and attenuate IFN-β expression. p21 therefore drives M1 to M2 macrophage reprogramming through a mechanism that promotes p50/p50 NF-κB prevalence and limits IFN-β production.

LPS-tolerant human monocytes upregulate p21

The immune response differs substantially between mice and humans, and several molecules that have a role in LPS tolerance in mice are not considered important mediators of LPS tolerance in humans (Biswas and Lopez-Collazo, 2009). Our next objective was therefore to evaluate the relevance of our findings in the human system, and to determine whether p21 has a role in LPS tolerance induction in human monocytes. To induce tolerance, monocytes isolated from healthy volunteers were LPS-tolerized (16 h), washed with PBS and rechallenged with LPS for various time periods (Cubillos-Zapata et al., 2014). Human monocytes showed a notable increase in p21 expression after LPS tolerization, or tolerization and rechallenge (Figure 31A, upper panel). IFN-β was downregulated considerably after LPS tolerization and rechallenge, compared to its strong induction after primary LPS activation (Figure 31A, lower panel). Monocyte hyporesponsiveness after LPS tolerization was also confirmed, as indicated by TNF-α downregulation and CCL2 upregulation in LPS-tolerized cells (Figure 31B). These experiments imply that p21 is a critical factor in regulating hyporesponsiveness and IFN-β expression in human monocytes. LPS tolerization of human monocytes has been associated with their polarization from an M1 to a M2 tolerant state (Porta et al., 2009); our data therefore suggest that p21 is a regulator of this reprogramming.

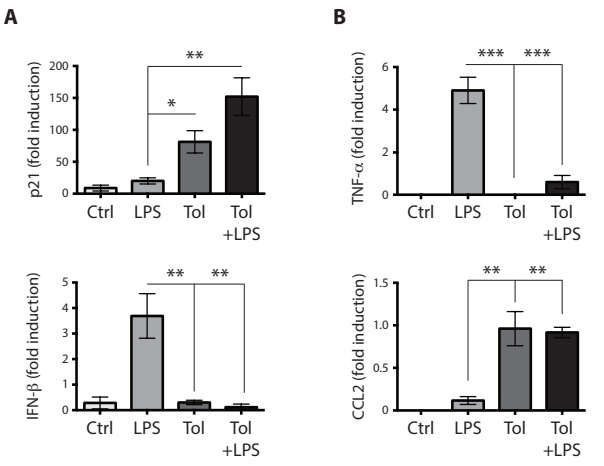


FIGURE 31 | Human monocytes upregulate p21 during *in vitro* LPS tolerance. Cultured human monocytes isolated from healthy volunteers were tolerized with LPS, washed, and restimulated with LPS. (**A**) RT-PCR analysis showing high p21 expression in tolerant human monocytes (upper). IFN-β expression was downregulated in tolerant cells (lower). (**B**) LPS-tolerant human monocytes showed significant downregulation of TNF-α and upregulation of CCL2 expression, compared with LPS-activated monocytes. Results were normalized to β-actin. Data shown as mean ± SEM (n = 4); *p<0.05; **p<0.01; ***p<0.001, one-way ANOVA with Bonferroni correction.

p21 knockdown reverses the LPS-tolerant status in human monocytes

To show directly that p21 contributes to induction of the hyporesponsive phenotype of human monocytes, we performed p21 small interfering RNA (siRNA)-silencing experiments in monocytes and studied their response to LPS after tolerization. RT-PCR confirmed that p21 mRNA was abolished in p21 siRNA-transfected LPS-tolerized monocytes (Figure 32A, upper panel). These monocytes showed a significant increase in IFN-β expression compared with control siRNA-transfected cells (Figure 32A, lower panel), which indicated that p21 downregulation allows IFN-β upregulation and halts monocyte hyporesponsiveness. In accordance with this observation, impaired hyporesponsiveness in p21 knocked-down monocytes was further confirmed by increased production of TNF-α and decreased production of the anti-inflammatory cytokine IL-10 (Figure 32B). These data identify p21 as a major participant in promoting LPS tolerance in human monocytes by limiting both IFN-β expression and their overall inflammatory status.

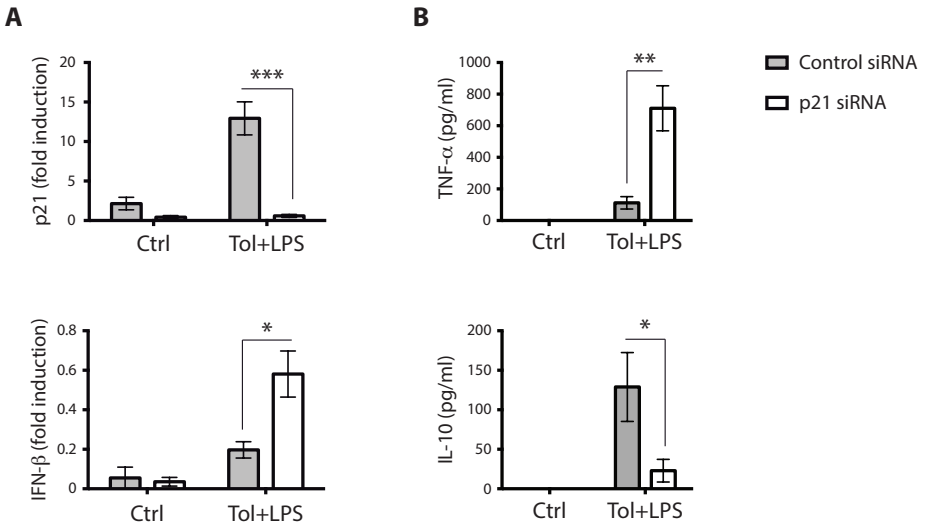


FIGURE 32 | **p21 knockdown reverts the LPS-tolerant status in human monocytes.** Cultured human monocytes isolated from healthy volunteers were transfected with p21 siRNA or control siRNA. Cells were then LPS-tolerized and -rechallenged. (**A**) After LPS rechallenge, RT-PCR showed abolished p21 mRNA in cultures transfected with p21-specific siRNA (upper) and upregulated IFN-β expression compared with control siRNA-transfected cells (lower). Results were normalized to β-actin. (**B**) Culture supernatants were analyzed for representative cytokine production by ELISA. In p21 siRNA-transfected tolerant human monocytes TNF-α production was significantly increased while IL-10 production was decreased, compared with control siRNA-transfected monocytes. Data shown as mean ± SEM (n = 4); *p<0.05; **p<0.01; ***p<0.001, two-tailed Student's t test.

p21 regulates human monocyte hyporesponsiveness during sepsis

Monocytes isolated from patients with sepsis show an immunosuppressed state and do not upregulate proinflammatory cytokines when challenged with LPS *ex vivo* (Faas et al., 2002; Escoll et al., 2003), a state that resembles LPS tolerance. Our final objective was to test whether p21 has a role in the hyporesponsive status of monocytes in sepsis.

Monocytes isolated from sepsis patients (clinical details in Table 1) expressed significantly higher p21 levels than healthy volunteers (Figure 33A, upper panel). This suggested that high p21 expression could control human monocyte hyporesponsiveness in sepsis and be associated with low IFN-β levels. Monocytes from sepsis patients showed an overall low IFN-β expression that correlated negatively with p21 expression in a statistically significant manner (Pearson correlation analysis). Patients with upregulated p21 had low IFN-β levels (Figure 33A, lower panel). Association of the hyporesponsive state of patient monocytes with low IFN-β expression was validated after an LPS challenge, which led to no IFN-β upregulation, whereas monocytes from healthy volunteers showed notable upregulation of this cytokine (Figure 33B, upper panel). p21 levels were high in monocytes from sepsis patients, and LPS treatment did not further increase p21 expression (Figure 33B, lower panel); this suggests that the already high p21 levels were sufficient to maintain unresponsiveness. The tolerant phenotype of monocytes in sepsis thus appears to be linked to high p21 levels, which account for the IFN-β reduction.

Since LPS-tolerant monocytes are considered analogous to hyporesponsive sepsis monocytes (Biswas and Lopez-Collazo, 2009), our results thus indicate a role for p21 in establishing the immunosuppressed state of monocytes from sepsis patients.

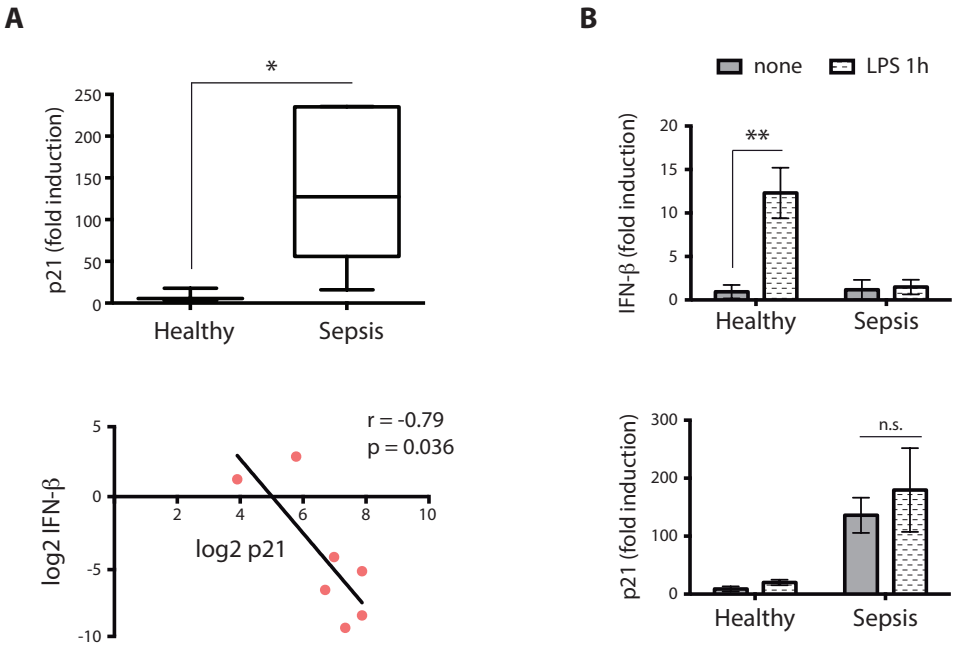


FIGURE 33 | p21 regulates hyporesponsiveness of human monocytes from sepsis patients. (**A**) Total RNA was extracted from monocytes isolated from sepsis patients and healthy volunteers and analyzed by RT-PCR. Upper, p21 mRNA levels were significantly higher in monocytes from sepsis patients compared with those from controls. Shown is median (n = 7); *p<0.05, two-tailed Mann-Whitney U test. Lower, Correlation between p21 and IFN-β levels in monocytes from sepsis patients. Results were normalized to β-actin and log2 values were used to calculate correlation; n = 7, two-tailed Pearson's test r = -0.7944, *p<0.05. (**B**) Monocytes from healthy volunteers and sepsis patients were challenged *ex vivo* with 10 ng/ml LPS (1 h). RT-PCR analysis showed IFN-β upregulation in monocytes from healthy donors but not from sepsis patients (upper). High p21 expression was detected in monocytes from sepsis patients before and after LPS treatment (lower). Results were normalized to β-actin. Data shown as mean ± SEM (n = 5); *p<0.05; **p<0.01; ***p<0.001, two-tailed Student's t test.

	Data (n = 7)
Age	68 ± 10.6 years
Cardiac frequency	106.2 ± 3.9 pulsations/min
Respiratory frequency	24.1 ± 1.9 breaths/min
Temperature	38.4 ± 0.4 °C
Creatinine	2.4 ± 0.3 mg/dl
Hematocrit	36.1 ± 3.7
Leukocytes	18894.6 ± 3674.9 cells/mm^3
Neutrophils	15910 ± 3674.9 cells/mm^3
Glucose	134.7 ± 47.7 mg/dl
K^+	4.8 ± 0.6 mEq/L
Na^+	138.5 ± 2.7 mEq/L
CO_3H	17.6 ± 1.2 mmol/L
Glasgow-CS[A]	13.9 ± 0.86
SAPS	12 ± 1
Microbiology	Gram-negative bacteria

[A] Glasgow Coma Scale is a neurologic scale that aims to give a reliable and objective way to evaluate the neurologic damage in patients. Glasgow-CS is part of the APACHE II score. The normal value of Glasgow-CS is 15 (a healthy-normal parameter matched with age). SAPS, simplified acute physiology score.

TABLE 1 | Clinical parameters.

DISCUSSION

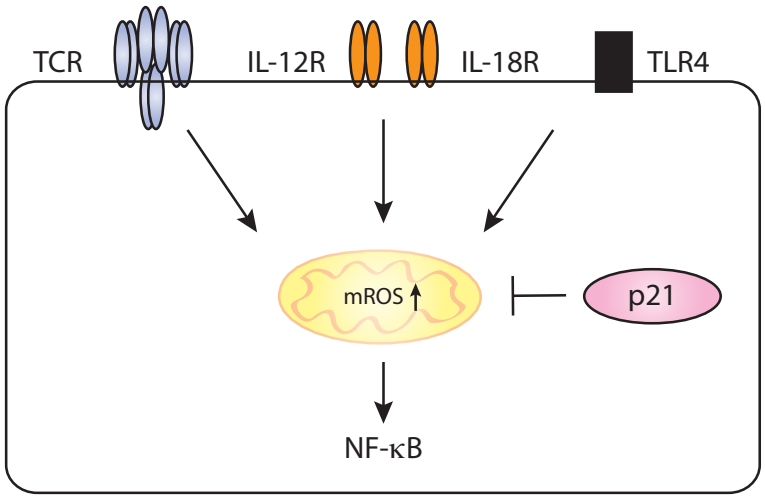
p21 as a regulator of immune homeostasis

As a small molecule able to participate in a number of specific protein-protein interactions, p21 has an essential role in the regulation of many biological functions such as cell cycle, apoptosis, cell differentiation and senescence, oxidative stress, and gene expression (Dotto, 2000). Ever since the initial discovery that p21-deficient mice develop lupus-like autoimmune disease (Balomenos et al., 2000), interest in p21-dependent regulation of immune responses has not abated. In the absence of p21, T cells hyperproliferate, leading to break of tolerance (Balomenos et al., 2000). p21 does not affect naïve T cells, but exclusively regulates the proliferation of effector/memory T cells that survive AICD (Arias et al., 2007). This property of p21 has a therapeutic effect, since p21 transgenic overexpression in T cells of B6/*lpr* mice prone to lupus-like disease reduced proliferation and activation of autoreactive T cells and ameliorated the disease (Daszkiewicz et al., 2015). In addition, reports from our laboratory and others demonstrated a role for p21 in regulation of innate immunity. In the absence of p21, macrophages show increased NF-κB activity and proinflammatory cytokine production in response to LPS, rendering the $p21^{-/-}$ mice extremely sensitive to septic shock (Trakala et al., 2009; Scatizzi et al., 2009). Furthermore, a p21 mimetic peptide had a therapeutic effect on macrophage activation during experimental inflammatory arthritis (Mavers et al., 2012).

These reports clearly link p21 with regulation of both innate and adaptive immune responses. The mechanism by which p21 regulates T cells and macrophages, as well as its impact on human patients, nonetheless remained unexplained. In this study, we establish that 1) p21 controls the activation of effector/memory T cells and their IFN-γ production by regulating mROS production, 2) p21 deficiency in LPS-activated macrophages leads to enhanced mROS production and DNA binding of p65/p50 NF-κB, and 3) p21 promotes macrophage reprogramming during LPS tolerance by regulating the balance between active p65/p50 and inhibitory p50/p50 NF-κB, which is relevant in human sepsis; p21 might thus be a target molecule for sepsis treatment.

p21 regulates IFN-γ production in effector/memory $CD4^+$ T cells through NF-κB

In B6/*lpr* mice, effector/memory T cells hyperproliferate and are hyperactivated, leading to development of lupus-like autoimmune disease (Balomenos et al., 1997; Fortner and Budd, 2005). We explored the p21 therapeutic effect by increasing its expression in B6/*lpr* mice. In addition to controlling T cell proliferation (Daszkiewicz et al., 2015), p21 transgenic expression reduced B6/*lpr* effector/memory T cell activation and the proportion of IFN-γ-secreting cells (Figures 1 to 3). These data suggest that overexpressed p21 has functions in T cell response control other than cell cycle inhibition. Overexpressed p21 did not affect the effector/memory T cell population of normal background B6-p21tg mice (Figures 1 and 2), indicating that endogenous p21 is sufficient to control normal T cell responses, whereas high p21 levels are needed to control autoimmune *lpr* T cells. *In vitro* study validated these results, and showed that after primary TCR stimulation



SCHEME 7 | p21 is a negative regulator of mitochondrial ROS. Through its effect on mitochondrial ROS, p21 regulates TCR-dependent and -independent T cell activation, as well as macrophage TLR4 activation, acting as a suppressor of inflammatory and autoimmune syndromes.

and IL-2 expansion, direct IFN-γ induction by IL-12 and IL-18 is significantly inhibited by the p21 transgene (Figure 3). This implied that p21 directly controls the IFN-γ induction pathway. Indeed, p21 deficiency in B6/*lpr* and B6 (WT) effector/memory T cells leads to increased IFN-γ induction by IL-12 and IL-18 (Figures 5 and 6). In addition, by reducing IFN-γ production in effector/memory T cells, the p21 transgene exerts a paracrine effect on macrophages, since the differential IFN-γ levels secreted by B6/*lpr* and B6/*lpr*-p21tg T cells greatly reduced macrophage activation and inflammatory potential (Figure 4).

In the absence of p21, effector/memory T cells produced elevated IFN-γ levels in response to stimulation with a combination of IL-12 and IL-18, but not with IL-12 alone (Figure 6). This suggested that p21 might control only IL-18-dependent IFN-γ induction. To verify this, we examined the activation of p-STAT4 and NF-κB, which are dependent on IL-12 and IL-18, respectively (Robinson et al., 1997; Nakahira et al., 2002). Contrary to our predictions, we found elevated activation of both STAT4 and NF-κB in $p21^{-/-}$ effector/memory T cells after IL-12/IL-18 stimulation (Figure 7). High activation of NF-κB and/or IFN-γ as induced by IL-18 could contribute to an increase in IL-12 receptor expression and consequently, STAT4 phosphorylation (Aita et al., 2004; Wu et al., 2000). We therefore suggest that lack of p21 enhances IFN-γ production in effector/memory $CD4^+$ T cells through its effect on IL-18-dependent NF-κB activation.

p21 regulates TCR-dependent and -independent effector/memory $CD4^+$ T cell activation through mitochondrial reactive oxygen species

Naïve, activated and memory T cells have different metabolic profiles (Ron-Harel et al., 2014). Sena et al. recently demonstrated a central role in signaling for mROS after antigen-specific T cell activation (Sena et al., 2013). In this study, mice with conditional deletion of the mitochondrial complex III subunit in T cells have decreased proportions of activated $CD44^{hi}CD62L^{lo}$ T cells, which show reduced IFN-γ production (Sena et al., 2013). Our study shows that p21 negatively regulates mROS production in effector/memory T cells, during both TCR- and IL-12/IL-18-dependent stimulation (Figures 8 and 9). Using DPI (diphenyleneiodonium), an inhibitor of mitochondrial complexes I and II, we demonstrated that mROS is necessary for IFN-γ production in effector/memory T cells (Figure 10). In confirmation of

these data, mROS inhibition strongly reduced p65 phosphorylation in $p21^{-/-}$ effector/memory T cells (Figure 13), which suggests that p21 negatively regulates NF-κB-dependent IFN-γ production through mROS (Scheme 7). Our results also showed that mROS is needed for $CD44^{hi}$ memory T cell generation (Figure 11). This finding could explain the fact that p21 deficiency leads to increased generation of effector/memory $CD44^{hi}CD62L^{lo}$ T cells (Figure 6).

p21 regulates mitochondrial reactive oxygen species production in macrophages

Mitochondrial reactive oxygen species (mROS) have long been considered harmful by-products of metabolism that damage cellular lipids, proteins and nucleic acids (Finkel and Holbrook, 2000). In the last decade, however, it has become evident that ROS are also crucial in cell signaling, in both innate and adaptive immunity. West et al. reported that TLR4 activation in macrophages leads to increased mROS production, which is needed for bacterial clearance (West et al., 2011a). In addition, cells from TRAPS (tumor necrosis factor receptor-associated periodic syndrome) patients show elevated LPS responsiveness due to increased mROS production (Bulua et al., 2011). Previous reports, including data from our laboratory, identified p21 as a negative regulator of NF-κB activity and proinflammatory cytokine production in LPS-activated macrophages (Trakala et al., 2009; Scatizzi et al., 2009). Increasing evidence indicates that mitochondrial ROS (mROS) can facilitate pro-inflammatory cytokine production through redox-sensitive activation of IKK complex and NF-κB (Bai et al., 2005; Gloire et al., 2006; West et al., 2011b); we therefore investigated whether p21 controls mROS in macrophages following LPS stimulation. At early times post-LPS stimulation, we found significantly increased mROS and cellular ROS in $p21^{-/-}$ mouse macrophages compared to those from WT mice (Figure 15). Lack of p21 did not affect MAPK or MyD88-independent pathways, indicating a role for p21 in negative regulation of TLR4 signaling through mROS (Scheme 7).

p21 promotes macrophage M1 to M2 reprogramming during LPS tolerance

Due to their plasticity, macrophages can polarize from an M1 to an M2 phenotype (Mosser and Edwards, 2008; Mills, 2012; Sica and Mantovani, 2012) or undergo functional reprogramming from an inflammatory to a hyporesponsive state (Shalova et al., 2015), while maintaining alternative function (Pena et al., 2011). Immunosuppressed monocytes and macrophages have been described in pathologies such as sepsis, acute coronary syndrome, and cystic fibrosis (Pena et al., 2014; Fernández-Ruiz et al., 2014; del Fresno et al., 2008). During sepsis development, macrophages undergo an intense proinflammatory response and also acquire a hyporesponsive state. Patient mortality is associated mainly to the immunosuppressed phase rather than to the inflammatory stage. Independently of its cell cycle inhibitory role, p21 inhibits LPS-induced proinflammatory macrophage activation and septic shock development (Trakala et al., 2009; Scatizzi et al., 2009). Here, we explored whether p21 might affect sepsis by controlling inflammatory macrophage reprogramming to a hyporesponsive phenotype. Since this hyporesponsive state resembles macrophage immunosuppression after LPS tolerization (Porta et al., 2009), we used this model

to study the role of p21 in the induction of hyporesponsiveness. LPS tolerance results in reprogramming of inflammatory M1 to M2-like macrophages that display a tolerant status after LPS rechallenge (Porta et al., 2009; Pena et al., 2011). We examined whether p21 affects the ability of macrophages to adopt a hyporesponsive phenotype and/or polarize to an M2 phenotype. Lack of p21 disrupted LPS macrophage tolerization, both *in vitro* and *in vivo*, and attenuated M1 macrophage reprogramming to an M2 hyporesponsive state. Analysis of this p21 effect led to three central conclusions. First, p21 promotes macrophage reprogramming to a hyporesponsive state by downregulating IFN-β. Second, p21 emerges as a regulator of the p65/p50 and p50/p50 NF-κB balance by promoting the accumulation of inhibitory p50/p50 complexes, which bind to the IFN-β promoter and downregulate IFN-β expression. Finally, p21 also governed human monocyte responses, as p21 silencing in these cells augmented IFN-β production and restricted LPS tolerization. These results support a system in which p21 upregulation and IFN-β downmodulation could contribute to hyporesponsiveness induction in monocytes from sepsis patients.

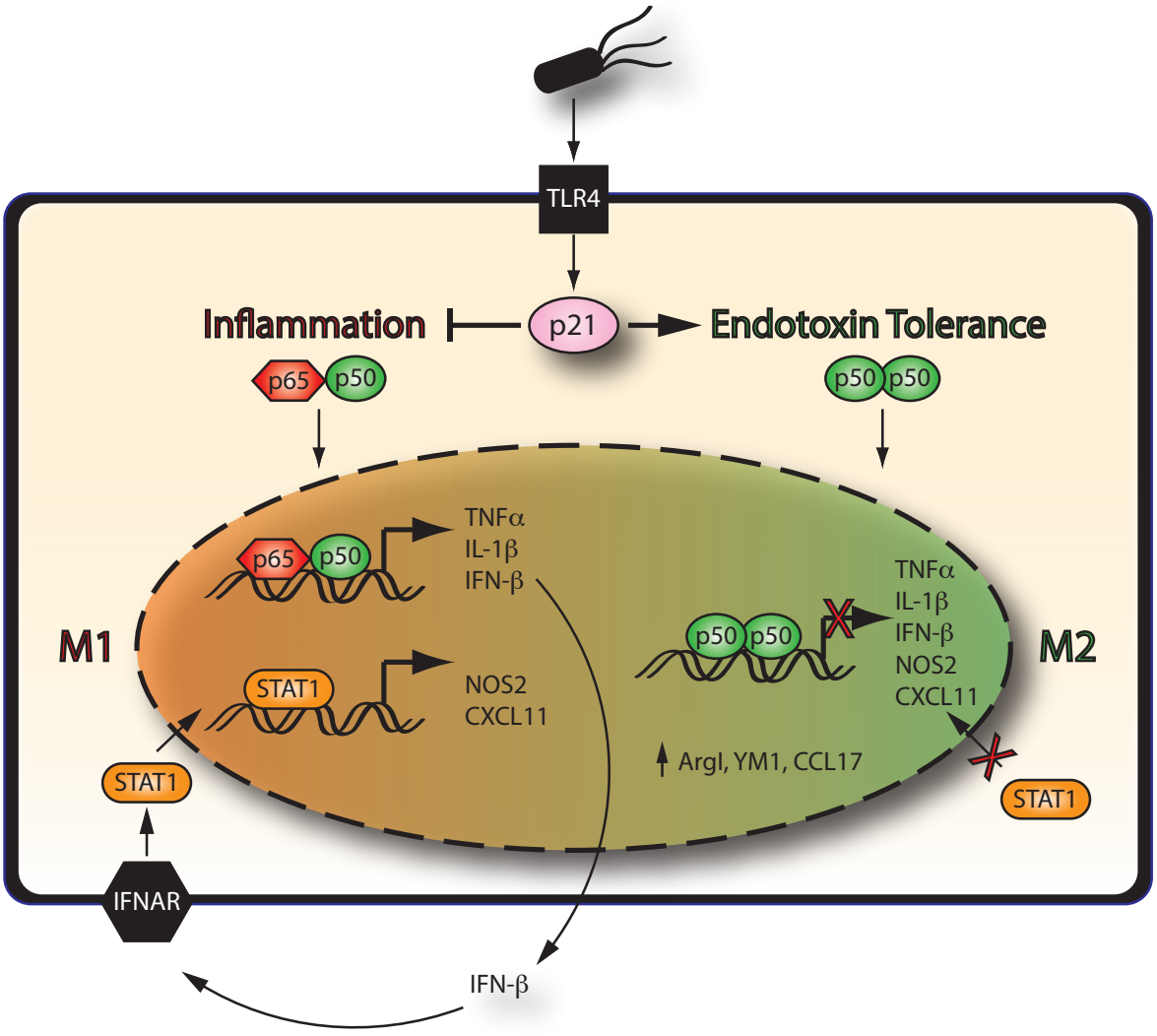
p21 deficiency impairs LPS tolerance

$p21^{-/-}$ mice do not develop tolerance after LPS priming and rechallenge, and die due to increased TNF-α and IFN-β production compared with WT mice. In the peritoneal cavity of $p21^{-/-}$ mice, we detected increased activation of M1-like $F4/80^{lo}$ macrophages (Ghosn et al., 2010; Ní Gabhann et al., 2014; Singh et al., 2015). Since M1 to M2 macrophage reprogramming is considered essential for *in vivo* LPS tolerance (Pena et al., 2011; Porta et al., 2009), our result suggested that compromised tolerance could be a result of failed M1 to M2 polarization. M2 macrophages, generated from M1 during LPS tolerance, are distinct from the conventional IL-4-dependent, alternatively activated M2 macrophages, which are not implicated in LPS tolerance (Rajaiah et al., 2013).

During *in vitro* LPS tolerization and rechallenge (Porta et al., 2009), $p21^{-/-}$ macrophages showed decreased expression of M2 markers compared with WT mice (Figure 23), while producing significantly higher levels the M1-associated cytokines TNF-α and IL-1β. Although TNF-α levels were clearly reduced after tolerization in both WT and $p21^{-/-}$ macrophages, IFN-β remained high after tolerization and prior to rechallenge only in the $p21^{-/-}$ macrophages. These persistent IFN-β levels, even in the absence of LPS stimulus, suggested that the p21 effect on IFN-β expression levels is of particular importance in tolerance induction. Concurring with this idea, IFN-β has been proposed to attenuate LPS tolerization, since it is expressed strongly in $p50^{-/-}$ macrophages, which fail to develop LPS tolerance (Porta et al., 2009).

p21-dependent IFN-β downregulation is crucial for development of LPS tolerance

During primary LPS treatment, p21 deficiency led to increased IFN-β production (Figure 17), although this did not translate into increased IFN-dependent gene expression, since IFN-β production in WT cells was sufficient to induce saturating IFNAR activation. In contrast, during LPS tolerization, WT cells downregulated IFN-β expression, while $p21^{-/-}$ cells produced significantly higher IFN-β levels (Figure 23), which led to increased STAT1 phosphorylation and IFN-β-dependent gene expression after LPS rechallenge (Figure 25). High IFN-β levels produced during primary LPS activation must



SCHEME 8 | **Model showing dual role of p21 in regulating macrophage activation and LPS tolerance.** During primary LPS activation, p21 suppresses the NF-κB activation pathway thus controlling proiflammatory cytokine and IFN-β production. After prolonged or repeated LPS stimulation, p21 controls the development of tolerance by promoting inhibitory NF-κB complex and the immunosuppression of LPS tolerant M2 macrophages.

thus be effectively downregulated for tolerance to occur; otherwise, sustained IFN-β autocrine stimulation maintains M1 proinflammatory status, obstructing tolerance. Since $p21^{-/-}$ macrophages produce high IFN-β levels after LPS tolerization and do not undergo M1 to M2 polarization, we considered that anti-IFN-β antibody treatment could enable tolerance development in the absence of p21. IFN-β neutralization during LPS tolerization allowed $p21^{-/-}$ macrophages to reach a hyporesponsive state like that of WT cells, with decreased M1 activation characteristics (Figure 26). Even when IFN-β was neutralized during LPS rechallenge, $p21^{-/-}$ macrophage responses such as STAT1 phosphorylation and iNOS induction were reduced. Increased IFN-β therefore appears to impair tolerance of $p21^{-/-}$ macrophages, which can be reestablished when IFN-β is blocked. This was further validated in an *in vivo* setting of LPS tolerance, in which neutralization of IFN-α/β receptor prior to LPS rechallenge radically improved the survival of $p21^{-/-}$ mice. These data indicate that p21 promotes macrophage reprogramming from a proinflammatory to a hyporesponsive state by downmodulating IFN-β production.

p21 regulates the balance between p65/p50 and p50/p50 NF-κB during LPS tolerance

As p21 negatively regulates NF-κB activation after primary LPS challenge (Trakala et al., 2009), we considered that p21 could also modulate this activation in rechallenged macrophages, and thus suppress M1 to M2 transition. In contrast to our predictions, $p21^{-/-}$ and WT macrophages showed similar NF-κB DNA binding activity after LPS rechallenge. Our data nonetheless identified a role for p21 in regulating the balance between active p65/50 and inhibitory p50/50 NF-κB dimers.

Accumulation of p50/p50 NF-κB was originally proposed to drive macrophage immunosuppression (Ziegler-Heitbrock, 2001); accordingly, $p50^{-/-}$ murine macrophages failed to develop LPS tolerance (Wysocka et al., 2001; Bohuslav et al., 1998). Furthermore, LPS tolerance, which induces M1 to M2 macrophage reprogramming, is driven by the increase of inhibitory p50/p50 over p65/p50 NF-κB dimers (Porta et al., 2009). In our system, lack of p21 impaired M1 to M2 macrophage reprogramming by sustaining low p50/p50 levels and a predomination of p65/p50 complexes. After secondary LPS stimulation, $p21^{-/-}$ macrophages showed a notable alteration of the relative expression pattern of p65/p50 *vs*. p50/p50 products bound to the NF-κB consensus sequence, with a reduction in p50/p50 bound complexes (Figure 30). This reduction accounted for the increased production of NF-κB consensus sequence-dependent products such as TNF-α. We further showed that after primary LPS stimulation, in which p21 regulates overall NF-κB activation, it also regulates the balance of p65/p50 and p50/p50 complexes later in the activation process (Figure 19). p21 thus has a dual role in NF-κB regulation; on the one hand, it suppresses the NF-κB activation pathway after LPS stimulation, and on the other, blunts NF-κB activity by promoting inhibitory NF-κB complex and immunosuppression of LPS-tolerant M2 macrophages (Scheme 8).

To determine how p21 deficiency drives IFN-β through regulation of the NF-κB complex, we examined the relative binding of p65/p50 *vs*. p50/p50 NF-κB to a specific IFN-β promoter sequence that differs from the NF-κB consensus sequence (Garoufalis et al., 1994). We detected remarkably increased p65/p50 binding to the IFN-β promoter sequence in $p21^{-/-}$ compared to WT LPS-rechallenged macrophages, while p50/50 binding was proportionally decreased. This result justified the increase in IFN-β by LPS-rechallenged $p21^{-/-}$ macrophages, and confirmed the role of p21 as a modulator of IFN-β production. In the absence of p21, the IFN-β promoter sequence showed higher p65/p50 and lower p50/p50 binding compared to the NF-κB consensus sequence. This finding could be explained by the fact that p65/p50 has greater affinity for the IFN-β promoter than for the consensus sequence, while the opposite is the case for p50/p50 (Frankenberger and Löms Ziegler-Heitbrock, 1997). Overall, these data indicate that p21 deficiency shifts the p65/p50 and p50/p50 balance toward the active p65/p50 NF-κB complexes, and that IFN-β expression is more susceptible to this p21 effect.

p50/p50 NF-κB accumulation during LPS tolerance depends on p65/p50 activation (Ziegler-Heitbrock, 2001). Our data, which show similar NF-κB pathway activation in both WT and $p21^{-/-}$ macrophages after LPS rechallenge, do not support upregulation of p50/p50 driven by p21-dependent p65/p50 accumulation. IL-10-dependent p50 stabilization by BCL-3 (Wessells et al., 2004; Carmody et al., 2007; Kuwata et al., 2003) might nonetheless be related to the p21 role as an enhancer of p50/50, since our results associate p21 deficiency with IL-10 downregulation (Figure 24). Our findings

showing that p21 modulates the p65/p50 and p50/p50 balance provide a clear advance in understanding this complex NF-κB regulation process.

p21 promotes LPS tolerance in human monocytes

Mechanisms that drive macrophage unresponsiveness differ between human and mouse models, and several molecules reported as mediators of LPS tolerance in mice (such as SHIP, SOCS1 and A20) (Sly et al., 2004; Liew et al., 2005) are not upregulated during hyporesponsiveness in humans (Del Fresno et al., 2009; Van 't Veer et al., 2007). It was therefore essential to verify whether p21 expression downregulates IFN-β expression and induces M2 hyporesponsiveness in human monocytes. After LPS tolerization and rechallenge, human monocytes showed increased p21 levels, which lowered IFN-β expression (Figure 31). This p21 function was validated directly, since p21 knockdown reverted the hyporesponsive phenotype of LPS-tolerized human monocytes, which had high IFN-β levels (Figure 32). In accordance with the analogous state between LPS tolerance and monocyte hyporesponsiveness in sepsis (Shalova et al., 2015), p21 was strongly upregulated in refractory patient-derived monocytes, while IFN-β was downmodulated (Figure 33). Statistical analysis showed a significant negative correlation between p21 and IFN-β levels. These data support the biological importance of our work and suggest that by reducing IFN-β levels, p21 is a key factor in driving macrophage hyporesponsiveness in human disease.

Although the IFN-β requirement has been established in viral infection, its role in bacterial infection is not clearly defined (McNab et al., 2015). In addition to its proinflammatory effect, an immunosuppressive role is also attributed to IFN-β (Yoo et al., 2014). The complex properties of this cytokine have been pointed out for inflammation and septic shock (Trinchieri, 2010).

Septic shock induction in mice requires IFN-β, as shown by studies with IFN-β and IFN-β receptor (IFNAR) knockout mice (Karaghiosoff et al., 2003; Huys et al., 2009), as well as other mouse models that regulate IFN-β expression (Mahieu and Libert, 2007; Mahieu et al., 2006). On the basis of these results, IFN-β targeting in human sepsis was proposed to have therapeutic potential (Mahieu and Libert, 2007). In contrast to its proinflammatory role, it is thought that IFN-β might trigger monocyte immunosuppression in sepsis or endotoxin tolerance (Biswas and Tergaonkar, 2007); in fact, recent experiments showed that IFN-β delivery severely reduces septic shock development in mice (Yoo et al., 2014). Due to the contrasting effects of IFN-β, the concept of limiting its signaling for therapeutic purposes entails conceptual difficulties. Our results, which show that p21-dependent IFN-β downmodulation drives monocyte hyporesponsiveness, discourage the use of IFN-β reduction-based treatment in sepsis, as it could further deteriorate responses to secondary infections.

Here we identified a role for p21 in driving mouse and human M1 to M2 macrophage/monocyte reprogramming, and established that upregulated p21 results in IFN-β downregulation and hyporesponsiveness in these cells. These findings link to our data from hyporesponsive monocytes of sepsis patients, which also showed increased p21 expression that correlated negatively with IFN-β levels. p21-driven IFN-β downregulation is therefore essential for induction of hyporesponsiveness in monocytes and macrophages. p21

modulates the p50/p50 and p65/50 NF-κB balance, allowing accumulation of inhibitory p50/p50 complexes that ultimately attenuate IFN-β production. These data demonstrate a role for p21 in fine-tuning the balance of NF-κB products and in promoting macrophage hyporesponsineness. This function differs distinct from its previously described role as a suppressor of the NF-κB activation pathway after macrophage stimulation. p21 is thus a major factor in potentiating p50-driven control of inflammation and a key molecule in regulating macrophage transition from a proinflammatory to a hyporesponsive state. Since regulation of this transition could be of therapeutic value for sepsis treatment, understanding this role of p21 could assist in defining molecular aspects of monocyte reprogramming and in discovering new targets for sepsis treatment.

CONCLUSIONS

1. In addition to controlling effector/memory T cell proliferation, p21 negatively regulates their activation and IFN-γ production. This role of p21 is physiologically relevant, since T cell-directed p21 overexpression reduces autoimmune disease manifestations in *lpr* mice by reducing IFN-γ production in effector/memory T cells. Conversely, p21 deficiency increases IFN-γ production in T cells from *lpr* mice.

2. p21 negatively regulates the production of mitochondrial reactive oxygen species (mROS) in effector/memory T cells, after both TCR-dependent and -independent stimulation. Through negative regulation of mROS, p21 controls NF-κB activation and IFN-γ production, as well as effector/memory T cell generation.

3. In macrophages, p21 acts as a negative regulator of TLR4 signaling through mROS and NF-κB, without affecting MAPK or MyD88-independent pathways. In addition to controlling overall NF-κB activity, p21 inhibits DNA binding of active p65/p50 NF-κB.

4. p21 is a key regulator of M1 to M2 macrophage reprogramming during LPS tolerance.

5. In LPS-tolerized macrophages, p21 promotes development of tolerance through IFN-β downregulation. This role of p21 has physiological relevance, since IFN-β-neutralization in p21-deficient mice improves survival following dual LPS challenge.

6. During LPS tolerance, p21 does not affect overall NF-κB activity; rather, it controls the balance between active p65/p50 and inhibitory p50/p50 NF-κB, by promoting accumulation of p50/p50 homodimers, which drive tolerance.

7. p21 drives LPS tolerance in human monocytes; it is upregulated in monocytes from sepsis patients, where it correlates negatively with IFN-β and might thus have therapeutic relevance.

RESUMEN EN ESPAÑOL

La proteína p21 participa en gran número de interacciones biológicas, entre las cuales se encuentra la regulación de muchos procesos celulares tales como la apoptosis, la diferenciación celular, el envejecimiento, el ciclo celular, el estrés oxidativo y expresión génica (Dotto, 2000). Su papel en las patologías autoinmunes se describió inicialmente al identificar una enfermedad autoinmune similar al lupus en ratones que carecían de este gen (Balomenos et al., 2000). Desde entonces nuestro grupo se ha centrado en descifrar su papel en la regulación de la respuesta inmune.

Inicialmente se identificó que su ausencia, conduce a una hiperproliferación de linfocitos T que se relaciona con una perdida de tolerancia (Balomenos et al., 2000). Sin embargo, p21 no afecta a los linfocitos T naive, pero regula exclusivamente la proliferación de linfocitos de memoria y efectores que sobrevivieron a AICD (Arias et al., 2007). Recientemente demostramos que esta propiedad de p21 tiene un efecto terapéutico, su sobreexpresión en linfocitos T de ratones B6/*lpr* con predisposicion para lupus redujo la proliferación y activación de linfocitos T autoreactivos y disminuyendo la gravedad de la enfermedad (Daszkiewicz et al., 2015).

Varios estudios incluyendo datos de nuestro grupo, demuestran que p21 participa en la regulación de la inmunidad innata. En su ausencia, los macrófagos muestran un incremento en la actividad de NF-κB y de producción de citoquinas en respuesta a LPS, haciendo los ratones $p21^{-/-}$ extremadamente sensible al shock séptico (Trakala et al., 2009; Scatizzi et al., 2009). En modelos de artritis inflamatoria la administración de un peptido similar a p21 tuvo un efecto terapéutico (Mavers et al., 2012); con estos hallazgos se relaciona a p21 en la regulación tanto con la inmunidad innata como con la adquirida. No obstante, no se ha descrito como es el mecanismo por el cual p21 regula la actividad de macrófagos y linfocitos T y su comportamiento en humanos:

Para nuestro estudio se establecieron los siguientes objetivos:

1. Investigar si el papel de p21 en la regulación de la activación de los linfocitos T $CD4^+$ de memoria y efectores es fisiopatológicamente relevante en el desarrollo de autoinmunidad.

2. Investigar si p21 regula directamente la producción de IFN-γ en linfocitos T $CD4^+$ de memoria y efectores.

3. Obtener un modelo explicativo de cómo p21 controla la activación de linfocitos $CD4^+$ de memoria y efectores junto con la producción de IFN-γ.

4. Estudiar el comportamiento de p21 en la activación de macrófagos dependiente de TLR4.

5. Investigar si p21, como un regulador negativo de la activación de TLR4, afecta la reprogramación de los macrófagos durante la tolerancia con LPS.

6. Identificar el mecanismo de cómo p21 regula la reprogramación durante la tolerancia de LPS.

7. Relacionar si los hallazgos obtenidos con p21 en la reprogramación de macrófagos durante la tolerancia con

LPS son relevantes en humanos durante procesos sépticos.

El reconocimiento de lo propio de lo no propio es una propiedad del sistema inmune que permite vivir en armonía con nuestras células y tejidos sin desarrollar enfermedades autoinmunes. Los ratones que tienen la mutación *lpr* desarrollan linfadenopatia y una enfermedad similar al lupus debido a la hiperactivación y proliferación de linfocitos T $CD4^+$, además, muestran acumulación de linfocitos T memoria y efectores los cuales producen grandes cantidades en IFN-γ, siendo esta una citoquina clave en el lupus. Estudios previos de nuestro grupo identificaron que p21 disminuye la autonimunidad al limitar la proliferación de los linfocitos T. Para investigar el efecto terapéutico de p21 en el lupus, sobre expresamos p21 en linfocitos T de ratones *lpr.* Como esperábamos la sobreexpresión de p21 redujo la expansión de linfocitos T memoria y efectores y síntomas de autoinmunidad. Nuestros resultados demuestran la capacidad de p21 para disminuir la producción de IFN-γ y la activación de linfocitos T. Estudios *in vitro* validan estos resultados, mostrando que la estimulación primaria de TCR, la expansión de IL-2, la inducción directa de IFN-γ por IL-12 y IL-18 fue inhibida significativamente por p21 transgénico. Por el contrario, la deficiencia de p21 aumenta la producción de IFN-γ en linfocitos T de memoria y efectores cuando son estimulados por IL-12 y IL-18, sugiriendo que p21 controla directamente la inducción de IFN-γ. En efecto, nuestros datos revelan que p21 controla inducción IFN-γ dependiente la estimulacion de IL-18 en linfocitos T de memoria y efectores a través de la regulación negativa de la actividad de NF-κB.

En la inmunidad contra patógenos intracelulares las especies reactivas de oxigeno (ROS) desempeñan un papel fundamental en el control de la infección y eliminación de microorganismos. Recientes estudios sugieren que los ROS en la mitocondria participan activamente en vías de señalización, tales como la activación de linfocitos T dependiente de TCR y activación de macrófagos vía TLR4. Por lo tanto encontramos que p21 controla la producción de ROS a nivel mitocondrial en los linfocitos efectores y de memoria cuando son estimulados via TCR e IL-12/IL-18. Este efecto de p21 es importante para la producción de IFN-γ, como inhibidor de ROS a nivel mitocondrial reduce de manera importante la activación de NF-κB y la producción de IFN-γ. Además la inhibición a nivel mitocondrial de ROS conduce a una disminución de la proliferación de linfocitos T de memoria y efectores. Estos datos exponen que la función de los linfocitos T de memoria y efectores está determinada de manera importante por los ROS a nivel mitocondrial e identificar a p21 como un regulador de la actividad tanto independiente como dependiente de TCR a través de los ROS a nivel mitocondrial. La información previa de nuestro grupo revelo que p21 regula de manera negativa la activación de NF-κB en macrófagos activados con LPS. Hasta aquí podemos identificar que p21 regula negativamente la producción de ROS en las mitocondrias cuando se estimulan los macrófagos con LPS. La carencia de p21 no afecta las vías de MAPK o MyD88, lo que indicaría que el papel de p21 en la regulación negativa of TLR4 es dependiente de la señalización por los ROS a nivel mitocondrial.

Los macrófagos pueden diferenciarse en dos fenotipos diferentes con funciones opuestas, están los que se activan de forma clásica (M1 o inflamatorios) y activados de manera alternativa (antiinflamatorios o M2). La plasticidad de los macrófagos es una propiedad extraordinaria, la cual le permite diferenciarse de fenotipo M1 a M2. En la sepsis, una causa importante de mortalidad, después de una intensa respuesta proinflamatoria, los

macrófagos M1 sufren una reprogramación funcional y adquieren un perfil de similar a su estado M2. Es importante destacar que la mortalidad está principalmente asociada con la fase de inmunosupresión debido al incremento de riesgo de infecciones secundarias. Los mecanismos moleculares subyacentes en la reprogramación de los macrófagos en sepsis no se han estudiado completamente, tal ves en estos mecanismos se encuentren nuevos avances terapéuticos. La reprogramación desde el fenotipo M1 a M2 puede ser simulada con la estimulacion repetida y prolongada con LPS cuando los macrófagos sufren tolerancia. Identificamos que p21 participa en la diferenciación de macrófagos de M1 a M2, independientemente de su papel como inhibidor del ciclo celular. La carencia en ratones de p21 no desarrollan tolerancia después la exposición repetida con LPS y mueren en relación a un incremento de mediadores asociados al fenotipo de macrófagos M1 e IFN-β . Los macrófagos $p21^{-/-}$ muestran alteraciones en la formación de homodimero p50/p50 NF-κB y su capacidad para regular la disminución de la producción de IFN-β y adquiere un estado de M2. Estos resultados revelan el papel fundamental en al regulación de IFN-β en procesos de inmunosupresión. En efecto, la neutralización de IFN-β permite a los macrófagos $p21^{-/-}$ alcanzar un estado de hiporespuesta al impedir su diferenciación a su fenotipo M1. Así, por medio de la regulación del balance entre su estado activo p65/p50 e inhibitorio p50/p50 NF-κB p21 favorece la acumulación de complejos p50/p50, los cuales se unen al promotor de IFN-β y disminuye su expresión. Los mecanismos que conducen a la hiporreactividad de los macrófagos difieren entre los modelos animales y humanos, por lo que es importante investigar si p21 afecta su diferenciación en humanos. *In vitro* la inducción de la tolerancia con LPS en monocitos humanos conduce a un significante incremento de la expresión de p21 lo que se relaciona con un perfil de diferenciación de estado M2 y disminución de la producción de IFN-β. Por el contrario en células donde se inhibe la expresión génica de p21 se revierte su estado de M2 y presentan incrementos de marcadores de fenotipo M1 y producion de IFN-β. Estos hallazgos confirma el papel de p21 en la diferenciación en monocitos humanos. También se ha identificado que en monocitos humanos de pacientes con sepsis, los cuales fueron extraidos en un estado de hiporespuesta, muestran un incremento en la expresión de p21 comparado con monocitos aislados de voluntarios sanos. Identificamos también una correlación negativa entre la expresión de p21 e IFN-β en pacientes con sepsis lo cual soporta el significado biológico de nuestro trabajo y sugiere que p21 es un factor substancial en la hiporreactivadad de los macrófagos en patologías humanas al reducir la expresión de IFN-β. En general toda esta información permite intuir un mecanismo de inmunosupresión de monocitos/macrófagos, a partir de la regulación de p21 del balance entre p50/p50 y p65/50 NF-κB favoreciendo la acumulación del dímero p50/p50 que induce la disminución de la producción de IFN-β y en consecuencia hiporreactividad. Por ultimo nuestros hallazgos designa a p21 como un regulador de la diferenciación de macrófagos en sepsis y como una posible diana terapéutica.

CONCLUSIONES

1. Ademas de controlar la proliferación de linfocitos T de memoria y efectores, p21 regula negativamente su activación y producción de IFN-γ. Esta función de p21 es fisiológicamente importante, en razón de que su sobreexpresión se relaciona con la disminución de la gravedad de las enfermedades autoinmunes en ratones *lpr* al reducir la producción de IFN-γ en linfocitos T de memoria y efectores. Por el contrario, la deficiencia de p21 incrementa la producción de IFN-γ en linfocitos T de ratones *lpr.*

2. A nivel mitocondrial en los linfocitos T de memoria y efectores la producción de especies reactivas de oxigeno (mROS) es regulado negativamente por p21, tanto de manera dependiente como independiente de la estimulación de TCR. A través de la regulación negativa de mROS, p21 controla la activación de NF-κB, la producción de IFN-γ, y la generación de linfocitos T de memoria y efectores.

3. En macrófagos, p21 participa como un regulador negativo de TLR4 realizando la señalización por medio de mROS y NF-κB, sin involucrar las vías de MAPK o MyD88. Además p21 controla actividad de NF-κB e inhibe la unión de p65/p50 NF-κB al DNA.

4. p21 es un regulador fundamental en la reprogramación de macrófagos de fenotipo M1 a M2.

5. En los macrófagos que se ha inducido tolerancia con LPS, p21 inhibe la expresión de IFN-β. Esta función es fisiológicamente importante debido a que la neutralización de IFN-β en ratones que carecen de p21 mejoran su supervivencia con la estimulación con LPS.

6. Durante la inducción de la tolerancia con LPS, p21 no afecta la actividad de NF-κB; lo que hace es regular el equilibrio entre su estado activo p65/p50 y su estado inhibitorio p50/p50 NF-κB, promoviendo la acumulación de p50/p50 que conduce a desarrollar tolerancia.

7. Encontramos que p21 induce tolerancia en monocitos humanos, al identificar que los monocitos de pacientes sépticos presentaban una correlación negativa con respecto al IFN-β y de esta manera tendría importancia médica al tener una posible diana terapéutica.

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SUPPLEMENTAL MATERIAL